

- (1) THE ONTOGENETIC EXPRESSION OF ANTIBODY VARIABLE-REGION
GENES IN THE CHICKEN
- (2) METHODS FOR FRACTIONATION OF PLASMA MEMBRANES AND MEM-
BRANE PROTEINS

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"G.O.D. does not play dice"

with apologies

Dedicated to my mother

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ABSTRACT

I. Immunoglobulins and antibodies made by chickens bursectomized in ovo on day 11 of incubation were studied by two-dimensional gel electrophoresis. It was found that bursectomized chickens have limited immunoglobulin diversity. Antibodies made by bursectomized responders were also of limited diversity. The L chains showed the greatest range in diversity restriction, followed by μ chains, while γ chains appeared normal. This limited diversity was stable over at least 20 weeks in that the same heavy and light chains were found over this time span. Thus bursectomy blocked the generation of immunoglobulin diversity in chickens.

Individual bursectomized chickens showed a range of extent of diversity restriction, presumably because the development of their bursal cells had been blocked at slightly different stages. Analysis of the particular heavy and light chains of total serum immunoglobulins, serum IgM, and antigen-specific antibodies made by individual bursectomized chickens showed that they were extensively shared. Statistical calculations indicate that if chickens have a normal repertoire of 200-300 genes coding for variable-regions of

L chains then it is very unlikely that random gene expression could result in such extensive similarities between bursectomized chickens. The implications of an orderly expression of variable-region genes are discussed.

II. Affinity density perturbation of erythrocyte ghosts with immunol latex microspheres was used to demonstrate the feasibility of this technique. Good agreement was obtained between experimental and theoretical results. Thus an analytical basis for the use of affinity density perturbation is established.

Isoelectric focussing was adapted to fractionate membrane proteins under non-denaturing conditions. This technique allowed the direct assay of rhodopsin in the gels by virtue of its spectral characteristics. It was shown also that this technique resolves rhodopsin from opsin, its end product on light absorption.

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INTRODUCTION

The Area-Code Hypothesis

Area-code molecules are cell-surface molecules which are involved in cell-cell interaction during growth and development. These molecules provide cells with distinct cell-surface phenotypes, and provide the basis for the specificity in cell-cell recognition during cell migrations and cell-cell interactions, as well as serving as receptors for diffusible differentiation signals. The area-code hypothesis has three major proposals: i) there is a progressive display of specific combinations of area-code molecules on the surfaces of cells during development; ii) the genetic programs which determine the specific expression of area-code molecules are in part controlled by DNA modifications. These chromosomal modifications are believed to channel cells into specific lineages with progressively restricted developmental options; iii) many of the area-code systems are organized into multigene families. A detailed discussion of the area-code hypothesis is presented in the appendix.

The proposal that DNA modification is a fundamental mechanism in programming development is likely to be the

most controversial of the theses embodied in the area-code hypothesis. Two major questions need to be answered if this proposal is to be credible. First, does DNA modification occur? A brief survey of the evidence for DNA modification in a variety of organisms will be presented in this introduction. Second, can DNA modification control gene expression in an orderly and precise fashion? Chapters I and II deal with the developmental readout of V genes of antibodies which are prototype area-code molecules and which employ DNA modifications. Finally, Chapters III and IV offer techniques for the purification and characterization of plasma membranes and membrane proteins.

Gene Expression and DNA Modification

There is a remarkable orderly unfolding of genetic information during development. From a single cell, the fertilized oocyte, a vast number of cells are generated. The cells are partitioned into lineages with precisely determined specializations. These specializations are the result of differential gene expression in different types of cells. One basic mechanism for differential gene expression is exemplified by the control of E. coli genes involved in sugar utilization. The genes and their products form interacting feedback loops such that the appropriate

genes are expressed, and others suppressed, in response to the particular sugars available in the environment (1).

Differential gene expression may also be achieved by DNA modification (2-4). The major reluctance to accept DNA modifications during development came from experiments of nuclear transplantation into amphibian oocytes (5). These experiments have certain limitations and should not be interpreted as showing that DNA modifications do not occur. It was found that in low frequencies cell nuclei from young tadpoles were capable of giving rise to fertile adult animals (6-8). However, it cannot be excluded that the few successes were due to contaminating germ-line cell nuclei or nuclei from stem cells reserved for the extensive tissue reorganization during metamorphosis. When nuclei from differentiated tadpole or adult cells were used development was invariably arrested at an early stage (8-11). The longest surviving embryos were arrested and died around the time of hatching. It appears that the defect in these embryos were not in the cells but in the organization of tissues (7). In fact, nuclei from an aneuploid cell line of adult liver origin supported development almost as well as nuclei from normal differentiated cells (8). This result points out the limited ability of nuclear transplantation experiments

to detect DNA changes. Thus nuclear transplantation experiments do not exclude the possibility of DNA modifications during development.

The modification of DNA has been found in prokaryotes as well as eukaryotes. Some viruses such as λ can undergo either a lytic or lysogenic life cycle. Each kind of life cycle utilizes an appropriate subset of the viral genes. The status of the whole genome, whether independent of or inserted into the host genome, determines which genes are to be expressed (12). The control of specific operons may also be controlled by DNA modification. The expression of one or the other flagellar gene in Salmonella appears to be controlled by the particular orientation of a reversibly invertible segment of DNA adjacent to one of the flagellar structural genes (13). Insertion sequences are DNA elements which can insert into and excise from bacterial chromosomes (14). Insertion sequences are normal constituents of E coli (15); whether they play a normal role in bacteria is uncertain, but their effects on bacterial gene expression is well documented. When insertion of the element occurs within a cistron the gene activity of the cistron may be affected. One insertion sequence, IS2, has opposite effects on gene expression depending on its

orientation when inserted in a cistron. In one orientation its effects resemble the other known insertion sequences in that the gene activity of the cistron is greatly reduced. In the other orientation gene expression may be constitutive, presumably due to transcription starting at a IS2 promoter (16). Transposons are larger chromosomal segments which behave much like insertion sequences (17). Indeed, their ability to transfer genes (e.g., antibiotic-resistance) may depend on terminally-placed inverted insertion sequences (18).

In maize, snapdragons, and Drosophila there are genetic instabilities which are very similar to those caused by insertion sequences (19-23). Gene activity may be affected by the insertion or excision of DNA elements, and by inversions, deletions, and transpositions. Individuals of species in many phyla have accessory chromosomes in addition to a normal diploid complement (24). The number of these chromosomes may increase or decrease. Their functions are unknown. With a few exceptions, there is no clearcut correlation of DNA modifications with normal developmental events in these eukaryotic systems.

Examples of developmentally correlated modifications of DNA are known. Chromosomal diminution is found in some

species of nematoda, copepoda and diptera (25-27). This is a process whereby parts of chromosomes or whole chromosomes are discarded in somatic cells. Chromosome fragmentation may accompany this diminution. The complete chromosomal complement is maintained only in the germ-cell line. The maintenance of the somatically discarded DNA may be obligatory for the development of germ cells. If these cells are experimentally induced to undergo chromosomal diminution, gametogenesis is defective. The abnormality is confined to the germ cell lineage, and the animals are sterile (27).

The alternate and mutually exclusive expression of genes determining mating types in Schizosaccharomyces may depend on reversible inversions (flip-flop) of a DNA segment between the two sets of genes (28). In addition, evidence was obtained showing that one set of genes (mat2^P) can be translocated into the other (mat1^m), thus masking the gene activity of the latter. The translocated mat2^P can be precisely excised and removed subsequently, with full restoration of mat1^m functions (29).

The most direct evidence for DNA modifications in controlling gene expression is found in the antibody multigene families. Embryonic and myeloma DNA were digested with restriction endonucleases. The DNA fragments were hybrid-

ized to myeloma L chain mRNA. It was found that the V and C genes were on the same myeloma DNA fragment, but on separate embryonic DNA fragments (30). Minimally, this indicated that the endonuclease site had been modified during differentiation. The embryonic DNA fragment containing the V gene was cloned and sequenced. The DNA sequences flanking the V gene showed no resemblance to the C gene (31). Thus two separate genes code for a single polypeptide (2, 32). Although formal proof for the joining of V and C genes must await the sequencing of the myeloma V-C gene fragment, it is very likely that the V and C genes are in fact joined at the DNA level. It would be very informative to determine the developmental readout of these genes.

Is V Gene Readout Orderly?

Any B cell expresses only one antibody. During development some mechanism must exist such that cells become committed to specific V genes (V_H , V_L) and C genes (C_H , C_L). The C_H genes were found to be read in the sequence C_{μ} first, then C_{γ} , followed by C_{α} (33). The order of readout of the other C_H genes is unclear, as is that of the C_{λ} genes. There is only one known C_{κ} gene.

The readout of V genes is much more difficult to study since there are many more V genes than C genes. Chickens

offer a convenient system, however, since the bursa of Fabricius is the central organ for B cell development. Bursectomy results in the deficiency of the humoral immune system: the number of B cells, the serum immunoglobulin levels, and the antibody responses to antigens are all affected (34, 35). Presumably the development of B cells was inhibited.

A classical ablation approach was taken to study the V gene readout in chickens. Embryos were hormonally bursectomized on day 11 of incubation, early in the differentiation of the B cell lineage. It was found that the resulting chickens have limited Ig diversity. The diversity did not detectably increase over at least 20 weeks. Thus bursectomy blocked the readout of antibody V genes (Chapter I).

A range of different extents of limited Ig diversity was found in individual bursectomized chickens, presumably because the generation of diversity had been blocked at slightly different stages. It is therefore possible to reconstruct the sequence of V gene readout by analyzing the particular H and L chains made by each chicken (Chapter II). The bursectomized chickens showed extensive similarities in the H and L chains they could make. Statistical calculations indicate that if chickens have 200-300 V_L genes then

it is unlikely that a random gene expression could result in such extensive similarities. The implications of an orderly readout are discussed.

The Cell Surface and Development: Technical Aspects

It was pointed out above that area-code molecules provide cells with distinctive cell-surface displays, and provide the basis for the specificity in cell-cell recognition. Many promising systems are available for the study of area-code molecules (see Appendix). Since many of the area-code molecules are expected to be integral membrane proteins, a biochemical approach in any of the systems may require the ability to purify and characterize plasma membranes and membrane proteins.

A novel method termed density perturbation was first suggested by Wallach et al. (36). The strategy is to attach particles with densities different from that of the target membrane so as to shift its density in a density gradient. The membrane is thus shifted away from its normal isopycnic position, and therefore away from any isopycnic contaminants. An adaptation was made to use the versatile latex microspheres developed by Molday & Dreyer (37) as the density perturbant (Chapter III). A model system (erythrocyte ghosts) was used to demonstrate the feasibility of this

approach. A theoretical framework was established for predicting the density shifts possible with different membranes and density perturbants. The method could be very useful since it combines the convenience of density gradient centrifugation with the versatility and specificity of the ligands attached to the latex microsphere.

A high resolution separation of membrane proteins can be achieved with isoelectric focussing. A method to do this under non-denaturing conditions was developed. Rhodopsin was chosen as a model membrane protein. The retention of the spectral characteristics of rhodopsin and its separation away from opsin was directly demonstrated. This technique is useful since the protein of interest may be assayed for directly by its known functional properties. It also provides a method for the purification of native membrane proteins for further functional studies.

REFERENCES

1. "The Lactose Operon". J. R. Beckwith and D. Zipser (eds.). Cold Spring Harbor Laboratory (1970).
2. Dreyer, W. J., and J. C. Bennett: The molecular basis of antibody formation: A paradox. Proc. Nat. Acad. Sci. (USA) 54:864-869 (1965).
3. Holliday, R., and J. E. Pugh: DNA modification mechanisms and gene activity during development. Science 187:226-232 (1975).
4. Sager, R., and R. Kitchin: Selective silencing of eukaryotic DNA. Science 189:426-433 (1975).
5. Briggs, R., and T. J. King: Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc. Nat. Acad. Sci. (USA) 38:455-463 (1952).
6. Gurdon, J. B., and V. Oerlinger: "Fertile" intestine nuclei. Nature (Lond.) 210:1240-1241 (1966).
7. Gurdon, J. B., and R. A. Laskey: The transplantation of nuclei from single cultured cells into enucleate frogs' eggs. J. Embryol. exp. Morph. 24:227-248 (1970).
8. Kobel, H. R., R. B. Brun, and M. Fischberg: Nuclear transplantation with melanophores, ciliated epidermal cells, and the established cell line A-8 in Xenopus laevis. J. Embryol. exp. Morph. 29:539-547 (1973).
9. Laskey, R. A., and J. B. Gurdon: Genetic content of adult somatic cells tested by nuclear transplantation from cultured cells. Nature (Lond.) 228:1332-1334 (1970).
10. Gurdon, J. B., R. A. Laskey, and O. R. Reeves: The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. J. Embryol. exp. Morph. 34:93-112 (1975).
11. Wabl, M. R., R. B. Brun, and L. DuPasquier: Lympho-

cytes of the toad Xenopus laevis have the gene set for promoting tadpole development. Science 190:1310-1312 (1975).

12. "The Bacteriophage Lambda". A. D. Hershey (ed.). Cold Spring Harbor Laboratory, New York (1971).
13. Zieg, J., M. Silverman, M. Hilmen, and M. Simon: Recombinational switch for gene expression. Science 196:170-172 (1977).
14. Starbinger, P.: IS - Elements in microorganisms. Crit. Rev. Microbiol.: in press (1977).
15. Saedler, H., and B. Hei : Multiple copies of the insertion-DNA sequences IS1 and IS2 in the chromosome of E. coli K-12. Molec. gen. Genet. 122:267-277 (1973).
16. Saedler, H., H. J. Reif, S. Hu, and N. Davidson: IS2, a genetic element for turn-off and turn-on of gene activity in E. coli. Molec. gen. Genet. 132:265-289 (1974).
17. Cohen, S. N.: Transposable genetic elements and plasmid evolution. Nature (Lond.) 263:731-738 (1976).
18. Ptashne, K., and S. N. Cohen: Occurrence of insertion sequence (IS) regions on plasmid deoxyribonucleic acid as direct and inverted nucleotide sequence duplications. J. Bacteriol. 122:776-781 (1975).
19. Fincham, J. R. S., and G. R. K. Sastry: Controlling elements in maize. Ann. Rev. Genetics 8:15-50 (1974).
20. McClintock, B.: The control of gene action in maize. Brookhaven Symp. Biol. 18:162-184 (1965).
21. Nevers, P., and H. Saedler: Transposable genetic elements as agents of gene instability and chromosomal rearrangements. Nature (Lond.) 268:109-115 (1977).
22. Fincham, J. R. S., and B. J. Harrison: Instability at the pal locus in Antirrhinum majus. II. Multiple alleles produced by mutation of one original unstable allele. Heredity 22:211-224 (1967).

23. Green, M. M.: Some observations and comments on mutable and mutator genes in Drosophila. Genetics Suppl. 73:187-194 (1973).
24. Muntzing, A.: Accessory chromosomes. Ann. Rev. Genetics 8:243-266 (1974).
25. Goldstein, P.: Chromatin diminution in early embryogenesis of Ascaris lumbricoides L. var. suum. J. Morph. 152:141-152 (1977).
26. Beermann, S.: The diminution of heterochromatic chromosomal segments in Cyclops (Crustacea, Copepoda). Chromosoma (Berl.) 60:297-344 (1977).
27. Bantock, C. R.: Experiments on chromosome elimination in the gall midge, Mayetiola destructor. J. Embryol. exp. Morph. 24:257-286 (1970).
28. Egel, R.: Frequency of mating-type switching in homothallic fission yeast. Nature (Lond.) 266:172-174 (1977).
29. Egel, R.: Rearrangements at the mating type locus in fission yeast. Molec. gen. Genet. 148:149-158 (1976).
30. Tonegawa, S., N. Hozumi, G. Matthysens, and R. Schuller: Somatic changes in the content and context of immunoglobulin genes. Cold Spring Harbor Symp. Quant. Biol. 41:877-890 (1977).
31. Tonegawa, S., C. Brack, M. Hirama, N. Hozumi, and G. Matthysens: Cold Spring Harbor Symp. Quant. Biol. 42: in press (1977).
32. Hood, L. E.: Two genes, one polypeptide chain -- fact or fiction? Fed. Proc. 31:177-187 (1972).
33. Lawton, A. R., P. W. Kincade, and M. D. Cooper: Sequential expression of germ line genes in development of immunoglobulin class diversity. Fed. Proc. 34: 33-39 (1975).
34. Kincade, P. W., K. S. Self, and M. D. Cooper: Survival and function of bursa-derived cells in bursec-

- tomized chickens. Cellular Immunol. 8:93-102 (1973).
35. Warner, N. L., J. W. Uhr, G. J. Thorbecke, and Z. Ovary: Immunoglobulins, antibodies and the bursa of Fabricius: Induction of agammaglobulinemia and the loss of all antibody-forming capacity by hormonal bursectomy. J. Immunol. 103:1317-1330 (1969).
 36. Wallach, D. F. H., B. Kranz, E. Ferber, and H. Fischer: Affinity density perturbation: A new fractionation principle and its illustration in a membrane separation FEBS Lett. 21:29-33 (1972).
 37. Molday, R. S., and W. J. Dreyer: New immunolatespheres: Visual markers of antigens on lymphocytes for scanning electron microscopy. J. Cell. Biol. 64:75-88 (1975).

CHAPTER I

Bursectomy Blocks the Generation of Immunoglobulin Diversity
in Chickens

Introduction

The anlage of the bursa of Fabricius in chickens appears on day 4-5 of incubation (1) and by day 7-8 bursal tissue histogenesis is complete and the bursa is receptive to the influx of blood-borne B stem cells (2-4). The bursa is the single central lymphoid organ for the differentiation of the humoral immune system (5-8). Stem cells are first demonstrable in 7 day bursal mesenchyme (2). The first surface IgM-positive cell is detected by immunofluorescence on day 12 in the bursa and on day 13 in the spleen (9, 10). Subsequently specific antigen binding cells could be detected in the lymphoid follicles: keyhole limpet hemocyanin (KLH)- and poly-L-(Tyr, Glu)-poly-D, L-Ala-poly-Lys (TGAL)-binding cells on day 16, and sheep erythrocyte-binding cells by day 18. Surgical, immunological, or hormonal bursectomy results in the specific impairment of the humoral immune system (11-16), presumably by blocking the development of B cell precursors.

There is indirect evidence that bursectomized chickens possess a limited diversity of B cell clones. For instance, bursectomized chickens may produce normal or supernormal amounts of immunoglobulin, but are deficient in their re-

sponse to antigenic challenge (6-8, 12, 17). Their serum immunoglobulins may show immunoelectrophoretic arcs of restricted mobility (17). The number of circulating B lymphocytes may be reduced (8).

We tested the heterogeneity of the immunoglobulin heavy (H) and light (L) chains of bursectomized chickens with a sensitive, high resolution assay. A two-dimensional high-resolution method has been described (18): isoelectric focussing first separates the proteins by their charge properties, followed by electrophoresis in a sodium dodecyl sulfate (SDS) gel with an exponential gradient of polyacrylamide to separate the proteins by their mobilities. Radioiodination is a convenient method for permitting very small quantities of proteins to be analyzed (19). Our results show that chickens bursectomized in ovo do indeed have limited immunoglobulin diversity which is stable over time, and imply that the developmental diversification of clones of B cells is blocked by bursectomy.

Materials and Methods

Eggs and chickens. - (UCD2 x UCD3)F₁ (20) fertile eggs were obtained from H. Abplanalp, University of California, Davis. Embryos were hormonally bursectomized on day 11 or 18 of incubation according to the procedure of Warner et al.

(7) except that the testosterone propionate solution in corn oil was sterilized by autoclave and emulsified with an aqueous solution of penicillin-streptomycin and amphotericin B (Gibco, Grand Island, New York) to 250U and 12.5 µg/ml, respectively. Controls received the same emulsion with the testosterone propionate omitted. Chickens were floor-reared on sawdust in an isolation room. Feed and water were ad libitum. The water was supplemented by addition of 25 ml/l molasses and 1 g/l Cosaterramycin (Pfizer, New York, New York) to prevent feces impaction (L. Schierman, New York Medical College, Valhalla, New York, personal communication).

Immunization. - All chickens were immunized starting at 6 weeks of age by six weekly injections of 1 mg human hemoglobin (Pentex, Kankakee, Illinois), 1 mg dinitrophenyl (DNP) -bovine gamma globulins (Calbiochem, La Jolla, California) per bird, subcutaneous and intra-muscular, emulsified in complete Freund's adjuvant (Gibco); and 2×10^7 sheep-erythrocyte ghosts, intravenous. Nine ml of blood was drawn into 1 ml 3.8% citrate 1 week after the last immunization. Cells were removed by centrifugation, the sera were delipidated by ultra-centrifugal flotation at 4° , and stored under liquid nitrogen until use.

Antisera. - Rabbit anti-chicken immunoglobulin antisera (Antibodies Incorporated, Davis, California) was purified by eluting specifically bound antibodies from a column of chicken immunoglobulins (21) coupled to Sepharose-4B (22). Rabbit anti-chicken μ chains were made by injecting rabbits with the IgM fraction purified by recycling of chicken anti-DNP antibodies on a Sephadex G-200 column (23) and further purified by passage through a column of chicken immunoglobulin-depleted sera (prepared by repeated passage through a rabbit anti-chicken immunoglobulin-Sepharose column) coupled to Sepharose, and then through a chicken IgG (21)-Sepharose column to render it μ -specific. The specificity was checked against a rabbit anti-chicken μ chain antiserum (gift of A. A. Benedict, Department of Microbiology, University of Hawaii, Hawaii). Goat anti-rabbit immunoglobulin antisera (Antibodies Inc.) was adsorbed against a chicken-sera-Sepharose column. Formalin fixed Staphylococcus aureus ATCC 12598 was a gift from J. Frelinger (Division of Biology, Caltech, Pasadena, California).

Radioiodination (19). - Fifty μ l Na^{125}I (New England Nuclear, Boston, Mass.) containing 2 mCi $^{125}\text{I}^-$ in isotonic phosphate buffer, pH 7.2, was added to 0.75 ml plasma previously dialyzed against isotonic phosphate buffer. One

hundred μ l 14 mM chloramine T was added and reacted at room temperature, 90 seconds. The reaction was terminated by the addition of 100 μ l 40 mM $\text{Na}_2\text{S}_2\text{O}_5$, and unreacted $^{125}\text{I}^-$ was removed by Sephadex G-25 desalting (24). Typically 40-80% incorporation was achieved.

Immunoprecipitation. - One hundred μ l rabbit anti-chicken immunoglobulin or μ chains was added to 100 μ l of the radioiodinated sera and incubated at room temperature, 30 minutes. An equivalent amount of goat anti-rabbit immunoglobulin was added to precipitate the antigen-antibody complexes. In some experiments 100 μ l of a 10% suspension of formalin-fixed staphylococci were used to bind the complexes. The precipitates or staphylococci were washed until the supernatants reached a background CPM level, and stored at -20° overnight.

Two-dimensional gel electrophoresis (18). - The immunoprecipitates were denatured by the addition of 10 μ l 2-mercaptoethanol, 100 mg urea (Ultra-Pure; Schwarz-Mann, Orangeburg, New York) and 50 μ l deionized water, and incubated at room temperature for 30 minutes. Another 50 μ l deionized water was added to dissolve all the urea, and the samples were incubated a further 90 minutes. 250,000 cpm samples were applied to the isoelectric focussing (first

dimension) gels. Staphylococci-bound immunoglobulins were similarly treated except that before use the solution was cleared of cells by centrifugation. For analysis of whole serum, 10 μ l 2-mercaptoethanol, 100 mg urea were added to 100 μ l sera, and incubated for 2 hours at room temperature. 0.7 OD₂₈₀ units were applied to the focussing gels. For analysis under denaturing but non-reducing conditions, 2-mercaptoethanol was omitted from the incubation mixture. For analysis under native conditions serum samples were directly applied to focussing gels (25); otherwise the procedure was as described (18). After electrophoresis the second dimension gel was fixed in 25% isopropyl alcohol, 10% acetic acid for 90 minutes, dried, and autoradiographs were made with Kodak NS2T Medical X-ray film. Sixty-hour exposure times were used. One week exposures were used to define the fainter spots. Further exposure, up to one ¹²⁵I half-life, did not reveal any new spots. Gels of whole sera were stained for protein (26).

Immunoabsorbent purification of antigen-specific antibodies. - Four hundred μ l or 300 μ l radioiodinated sera were added to 10^8 sheep erythrocytes (lightly fixed with 0.25% glutaraldehyde, antigenic properties were preserved as judged by hemagglutination assay) or 20 μ l packed DNP-

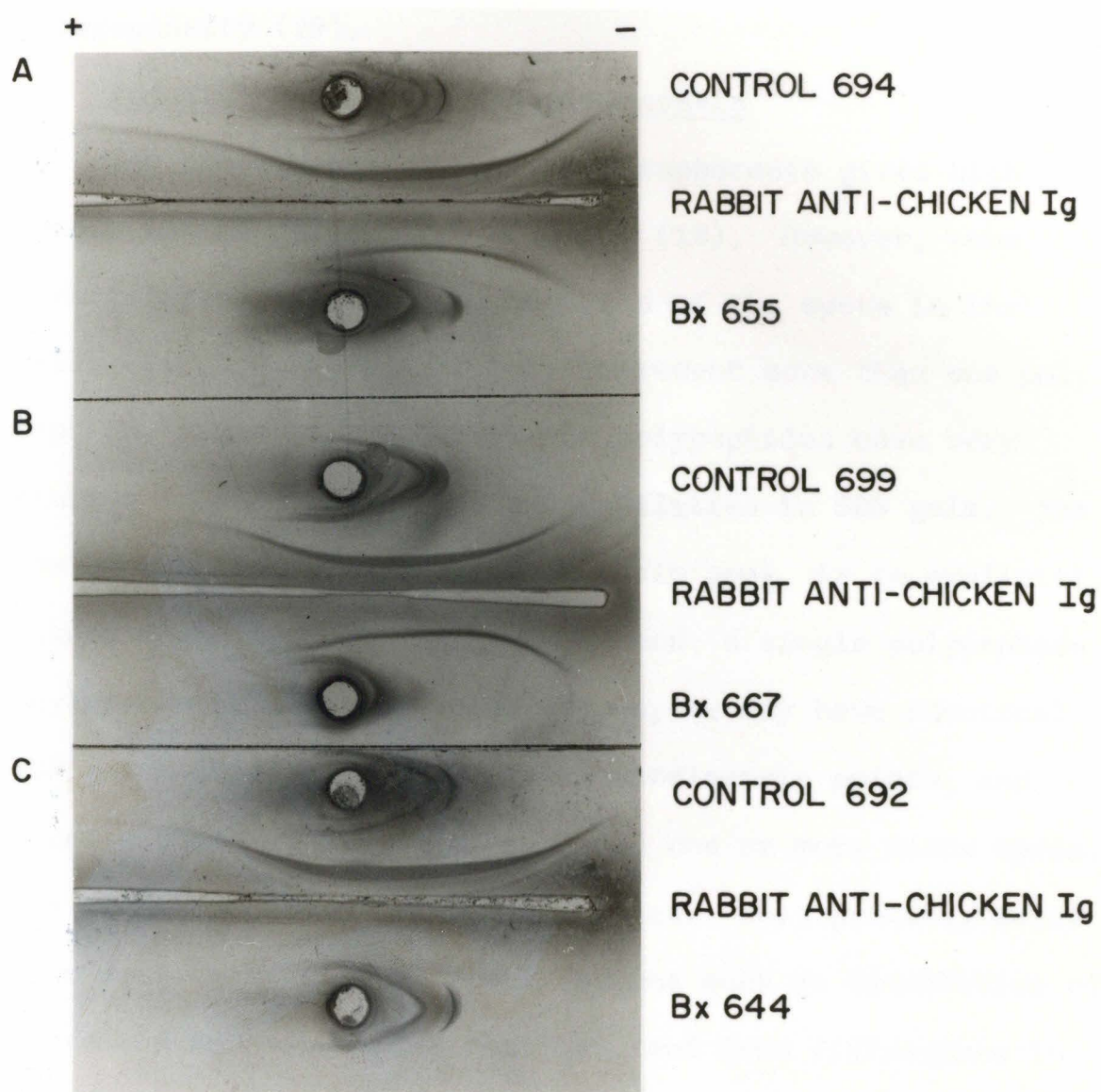
lysine-Sepharose, respectively, and incubated at room temperature for one hour. The adsorbents were washed, the antibodies were eluted by reduction and denaturation, and electrophoresed as above.

Other methods. - Hemagglutination was performed with the Microtiter system (Cooke Laboratory Products, Alexandria, Virginia). Immuno-electrophoresis and radial immunodiffusion quantification of serum immunoglobulin concentration were as described (27, 28). N-terminal amino acid sequence determination was made using a microsequenator (D. B. Helphrey and W. J. Dreyer, in preparation).

Results

In agreement with previous reports (6, 7) chickens bursectomized on day 11 of incubation were hypogammaglobulinemic (mean serum immunoglobulin concentration = 6% of mean control level, range 0.1-24%), and were deficient in their response to repeated antigenic challenge. Three of seventeen bursectomized chickens responded to sheep erythrocytes with titers of 2-3 while controls had titers of ≥ 6 . Immuno-electrophoretic screening of sera from bursectomized chickens showed that their IgG arcs, when detectable, were of diminished widths. In some cases the arcs were relatively cathodally displaced (17) (figure 1). This kind of

Figure 1. Immunoelectrophoresis of sera from bursectomized chickens. Rabbit anti-chicken immunoglobulin was used to precipitate the IgG. 655 (A) and 667 (B) were representative of the bursectomized chickens in having IgC arcs of decreased width and relatively cathodal migration. The IgG arc of chicken 644 (C) was undetectable. Nonspecific precipitation arcs close to the sample application wells were present in all samples. IgM was not detectable under these conditions. Bx, bursectomized chicken.



pattern could be interpreted as evidence for limited IgG heterogeneity (29).

I. Two-dimensional gel electrophoresis

The two-dimensional gel electrophoresis gives high resolution of polypeptide mixtures (18). However, some uncertainties exist in the analysis of the spots in the gels. First, a given spot may represent more than one polypeptide. This will occur if the polypeptides have very similar isoelectric points and mobilities in SDS gels. The use of the term, for example, L chain spot, is to explicitly acknowledge this uncertainty. Second, a single polypeptide may give more than one spot. The spots may have identical SDS gel mobilities but different isoelectric points, and usually there are one major spot and one or more minor spots. The minor spots are termed satellites. They probably arise from post-translational modifications such as deamidation of glutamine and asparagine residues, and from differences in glycosylation with charged sugars (e.g., sialic acid). In other cases the spots, usually two, differ only in their SDS mobilities. This is probably due to a more extensive glycosylation (23) in the spot with lower mobility. Because of these uncertainties the absolute number of H and L chains in the chickens is unknown. However, the relative diversity

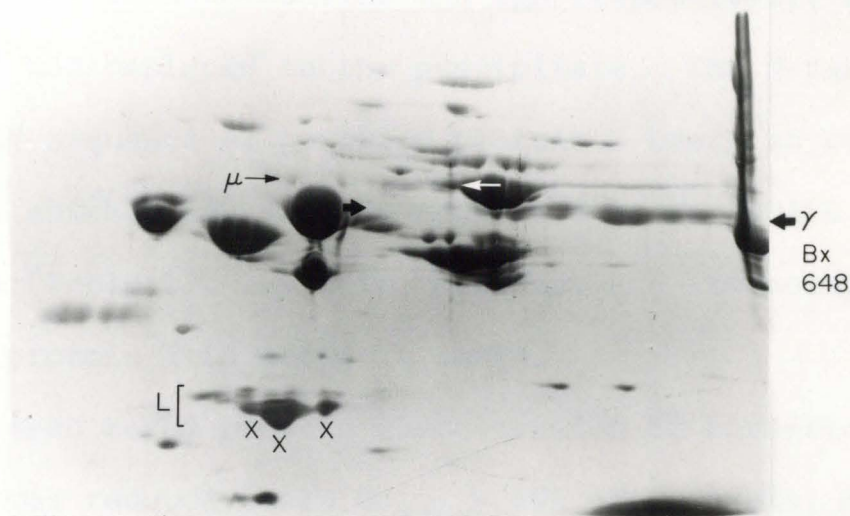
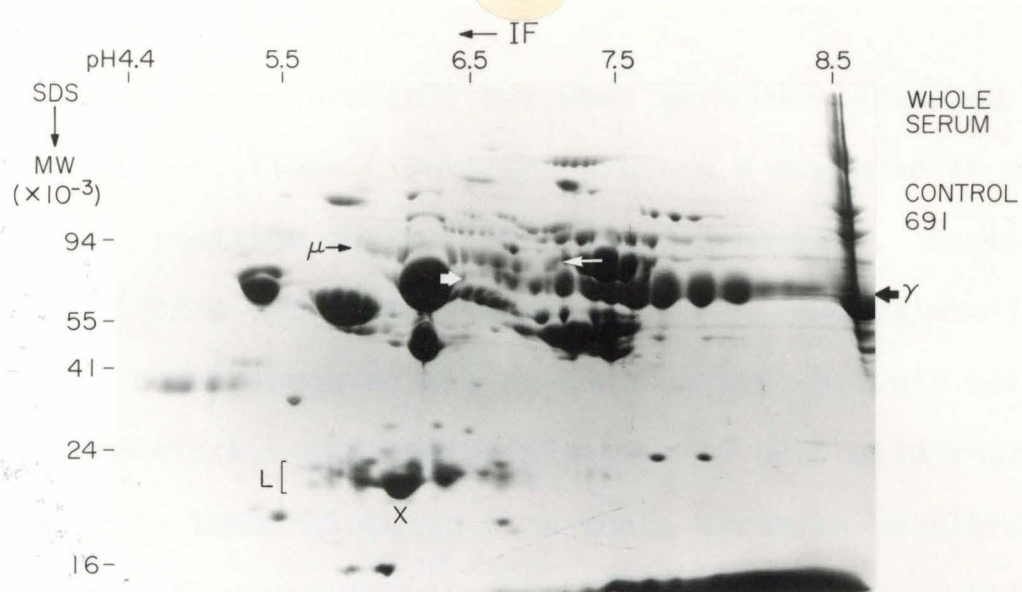
between chickens can be estimated by comparing the number of spots in each chicken, since it is reasonable that similar post-translational processes exist in all the chickens.

II. Two-dimensional map of whole sera. - On two-dimensional electrophoresis, reduced and denatured normal chicken sera and immunoglobulins purified from pooled sera (21) gave a heterogeneous pattern of spots in the μ (MW = 68,000 21 pI = 6.3-8.5), γ (MW = 71,000 pI = 5.7-7.6) and L chain (MW = 22,000-24,000 pI = 5.5-7.5) regions (figure 2a). The bursectomized chicken with the highest concentration of serum in immunoglobulin showed much fainter μ and γ chain spots, and a restricted set of L chain spots (figure 2b). The other bursectomized chickens had either very faint μ and γ spots and no detectable L spots, or no detectable immunoglobulin spots at all (figure 2c).

All maps showed an intense spot (dubbed protein X) usually associated with two satellites, which had pI and SDS mobilities similar to L chains. Protein X is not an L chain. In its native state it is a non-disulfide bonded multimer (molecular weight >70,000), not associated with any heavy chains. In the maps of whole sera it does not co-migrate with L chains if in the first dimension the sera were denatured and not reduced (immunoglobulins migrate as

Figure 2. Two-dimensional map of reduced and denatured whole sera of a control (691), and two bursectomized chickens (648, 669) stained for protein. Chicken 648 had the highest immunoglobulin L chain diversity of the bursectomized chickens. Chicken 669 is representative of the others. μ (thin arrows), γ (thick arrows), L indicate positions where purified immunoglobulins migrate to under the same conditions. IF isoelectric focussing, first dimension; SDS, exponential gradient gel, second dimension; X, Protein X (see text). Identical amounts of protein from control and bursectomized chickens were used; since bursectomized chickens had much decreased immunoglobulin levels the non-immunoglobulin spots in the maps of sera of bursectomized chickens are relatively more intense than those of controls.

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whole denatured molecules), nor when sera were focussed under native conditions. Purified protein X migrated in the prealbumen position during immunoelectrophoresis. Repeated passage of sera over a rabbit anti-chicken immunoglobulin-Sepharose column removed all immunoglobulins but did not affect protein X. Immunoprecipitation of sera with rabbit anti-chicken immunoglobulin or μ chain antisera resulted in enrichment of immunoglobulins and IgM respectively, while protein X was depleted in the precipitate. The N-terminal amino acid sequence of purified protein X bears no resemblance to chicken (30-32) nor mouse and human (33) L chain V-regions (Table 1). By both molecular and immunological criteria protein X is not an L chain.

The mean serum protein concentration of bursectomized chickens was reduced (mean $OD_{280} = 60\%$ of controls, range 45-80%). This is due to the decreased immunoglobulin levels in the bursectomized chickens. In the maps no other qualitative differences were seen between bursectomized chickens and controls apart from the immunoglobulin spots. However, differences in serum proteins present at concentrations below the sensitivity limit of this procedure could not be excluded. The sensitivity could not be increased any further by higher serum loading since the major non-immunoglobulin

Table 1

	1*			5					10				15			
Protein X				Asp	Glu	Pro	Gln	Aspt†	Glu	Pro	Aspt†	Arg	-	Leut†	-	Asp
								His			His			Ile		
								Glu								
Chicken λ				Ala	Leu	Thr	Gln	Pro	Ala	Ser	Val	Ser	Ala	Asx	Pro	Gly Glx
Human λ	Glp	Ser	Val	Leu	Thr	Gln	Pro	Pro	Ser	Val	Ser	Gly	Thr	Pro	Gly	Gln
Mouse λ	Pca	Ala	Val	Val	Thr	Gln	Gln	Ser	Ala	Leu	Thr	Thr	Ser	Pro	Gly	Glu

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Table 1. N terminal Amino Acid Sequence of Protein X.

The N-terminal 13 residues of Protein X is compared to N-terminal sequences of λ chains of chicken (30-32), human Ha, and mouse 104E (33).

* Alignments and numbering from (33)

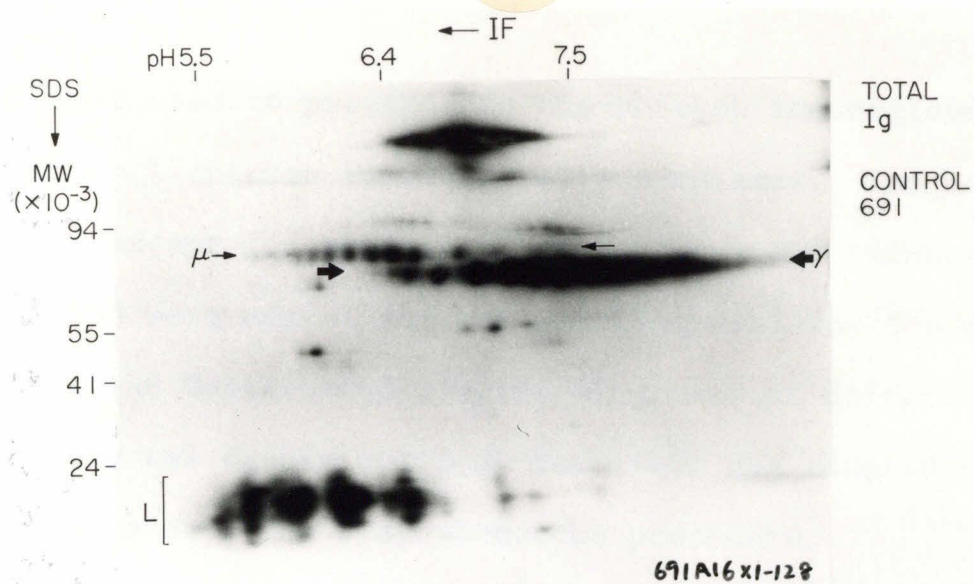
† At these positions alternate residues were detected

spots (e.g., albumen) become grossly overloaded, leading to artifacts. For the same reasons it was not possible to compare the immunoglobulin diversity of most of the bursectomized chickens using this approach because they had very low amounts of immunoglobulins.

III. Map of immunoprecipitation-purified radiolabeled immunoglobulins. - To improve on the detection of immunoglobulin spots, sera were radioiodinated to very high specific activities (ca. 1 mCi/ml sera). The immunoglobulins were purified by immunoprecipitation with rabbit antibodies specific for chicken immunoglobulins. The purified immunoglobulins were mapped by two-dimensional electrophoresis and autoradiography. Under the conditions used, 75 ng (1 pmole) of a γ chain was enough to give a single intense spot on the autoradiograph. The sensitivity limits, determined by maps of serial dilutions of control sera with an immunoglobulin-depleted serum, were 10 and 30 μ g/ml for IgM and IgG respectively. At the sensitivity limit the spot pattern of immunoglobulins from normal chickens is clearly distinguishable from those of bursectomized chickens. A reproducible pattern of co-precipitating serum proteins was seen in the maps (figure 3). These contaminants were totally removed if formalin-fixed protein A-bearing staphylococci

Figure 3. Two-dimensional autoradiographic (6-hour exposure) map of immunoprecipitation-purified total immunoglobulins of a control (691), and two bursectomized chickens (648, 644). Chicken 648 had the highest immunoglobulin L chain diversity of the bursectomized chickens, and chicken 644 had the lowest immunoglobulin L chain diversity. (thin arrows), (thick arrows), L represent the immunoglobulin spot positions. Note that coprecipitating non-immunoglobulin spots are present; these can be removed by substituting the goat anti-rabbit immunoglobulin antisera with staphylococci in the immunoprecipitation procedure, but are useful as internal markers (see text). Identical samples of 250,000 cpm were used for all the gels; since bursectomized chickens had much reduced immunoglobulin levels, the non-immunoglobulin spots were relatively more intense than corresponding spots of the controls, and therefore the sensitivity for the immunoglobulin spots of bursectomized chickens was also increased over that for control spots. When the sensitivity was adjusted to be equal by longer exposure of the control gels the L chain pattern is identical to that of chicken 648.

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(34) were used to precipitate the chicken immunoglobulin-rabbit anti-chicken immunoglobulin complexes. The contaminants, except for protein X, do not mask the immunoglobulin spots and were useful as internal landmarks for the maps obtained, and facilitated precise alignment of different maps. Therefore the double antibody technique was adopted in preference over the staphylococcus procedure.

The great increase in sensitivity allowed the detection of immunoglobulins of bursectomized chickens (figure 3). All bursectomized chickens gave spots in the μ , γ , and L regions. These were genuine immunoglobulin spots since under the same conditions an immunoglobulin-depleted serum showed only three spots in the L chain region, representing contaminating protein X, and no spots in the μ and γ regions. In analyzing the number of immunoglobulin spots in the maps, all spots appearing in the maps of the immunoglobulin-depleted serum were considered as non-immunoglobulin. When a chicken had an intense L chain spot which on alignment with other maps overlay an area which contained many spots in the other chickens, the intense spot was considered to be the aggregate of all those spots. This procedure tends to overestimate the number of spots in a chicken with intense L chain spots. The procedure errs on the conservative

side since it gives a maximum estimate of the spot diversity.

By making all pairwise comparisons of the maps, the number of H and L chain spots in each chicken was determined. The largest range in diversity was found in the L chains (Table 2). The diversity increased from a few very faint spots to one indistinguishable from that of the controls. The diversity of L chains appeared greater than that of H chains. Part of this could be because the resolution of the H chain region was less than that of the L chain region due to the logarithmic relationship of mobility versus molecular weights in SDS gel electrophoresis. All but one of the bursectomized chickens had less diverse L chain spots than controls. It was also noted that L chain spots showed the largest intensity differences. A faint spot in one chicken may be very intense in another. This probably reflects quantitative differences.

In contrast to the discrete small spots seen in most of the bursectomized chickens, large diffuse spots were seen in chicken 648 (which had the greatest L chain spot diversity), in chickens bursectomized on day 18 and in controls. This was interpreted as the fusion of many different spots, the resolution of the procedure being inadequate for the high

Table 2. - Number of H and L chain spots.

The number of μ , γ , and L chain spots in each chicken were determined by making all pairwise comparisons of the maps.

* The IgM maps were less sensitive than the total immunoglobulin maps; very faint L chain spots in the latter were undetectable in the former.

† ND not determined.

§ Spots were intense and diffuse. No accurate spot number counts could be made. Chicken 648 was estimated to have at least 83 L chain spots in the total immunoglobulin map.

Table 2

Chicken	L chains		γ	μ
	Total Ig map	IgM map*	Chains	Chains
Bx 644	14	16	3-4	18
672	17	9	6	18
669	24	8	9	16
670	32	11	10	18
664	39	22	11	18-19
636	41	ND†	9	ND†
655	42	29	9-10	18
671	43	33	12-13	18-19
667	45	19	8-9	18-19
668	48	37	13	18-19
666	49	19	9-10	18
676	52	25	12	18
673	53	41	9-10	17
655	56	26	9-11	18
628	58	39	9-10	18-19
648	> 58§	> 56§	12	17-18
Control 691	> 58§	> 56 §	13-14	18
692	> 58§	> 56§	14	17-18
693	> 58§	ND	14-19	ND
694	> 58§	ND	17	ND

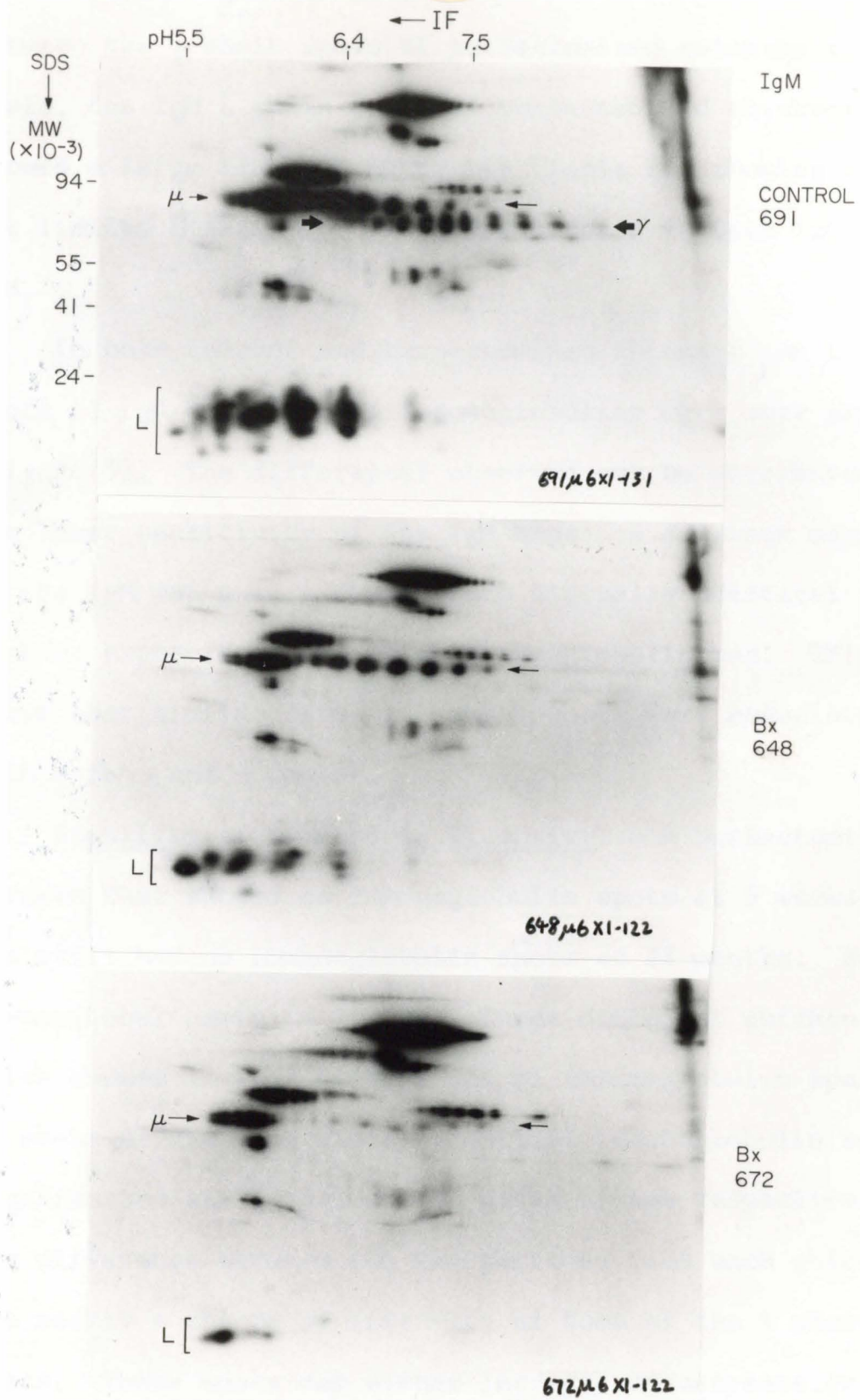
immunoglobulin diversity in the latter animals.

The γ chain spots of bursectomized chickens were less diverse than those of controls. The differences in γ chain spots between individual chickens were less dramatic than the differences seen in the L chain region. The γ chain spots of all bursectomized chickens were also less intense than those of controls.

A quantitative but not qualitative difference was seen between bursectomized chickens and controls in the μ region. The spots were fainter in the bursectomized chickens. This was confirmed when serum IgM was isolated and mapped (figure 4). The sensitivity limit for detection of IgM was 20 $\mu\text{g/ml}$ using the μ -specific antisera. A 136 ng (2 pmoles) sample of μ chain was enough to give an intense spot. The μ chain spots of bursectomized chickens were as diverse as the controls. A consistent spot intensity difference was seen. While the most intense μ spots of bursectomized chickens were of two groups of pI 5.8-6.2 and pI >7.0, controls had the most intense spots between pI 5.6 and 7.0. The μ chain spots of chicken 648, which had the highest diversity of L chain spots, resembled controls in having more intense acidic μ spots. Its spots were fainter than controls.

Although little or no difference in diversity was seen

Figure 4. Two-dimensional autoradiographic (60-hour exposure) map of immunoprecipitation purified IgM of a control (691), and two bursectomized chickens (648, 672). Chicken 648 had the highest IgM L chain diversity of the bursectomized chickens, and chicken 672 had the lowest IgM L chain diversity. μ (thin arrows), γ (thick arrows), L represent the immunoglobulin spot positions. Some IgG was copurified and detected in the control bird. Identical samples of 250,000 cpm were used for all the gels.



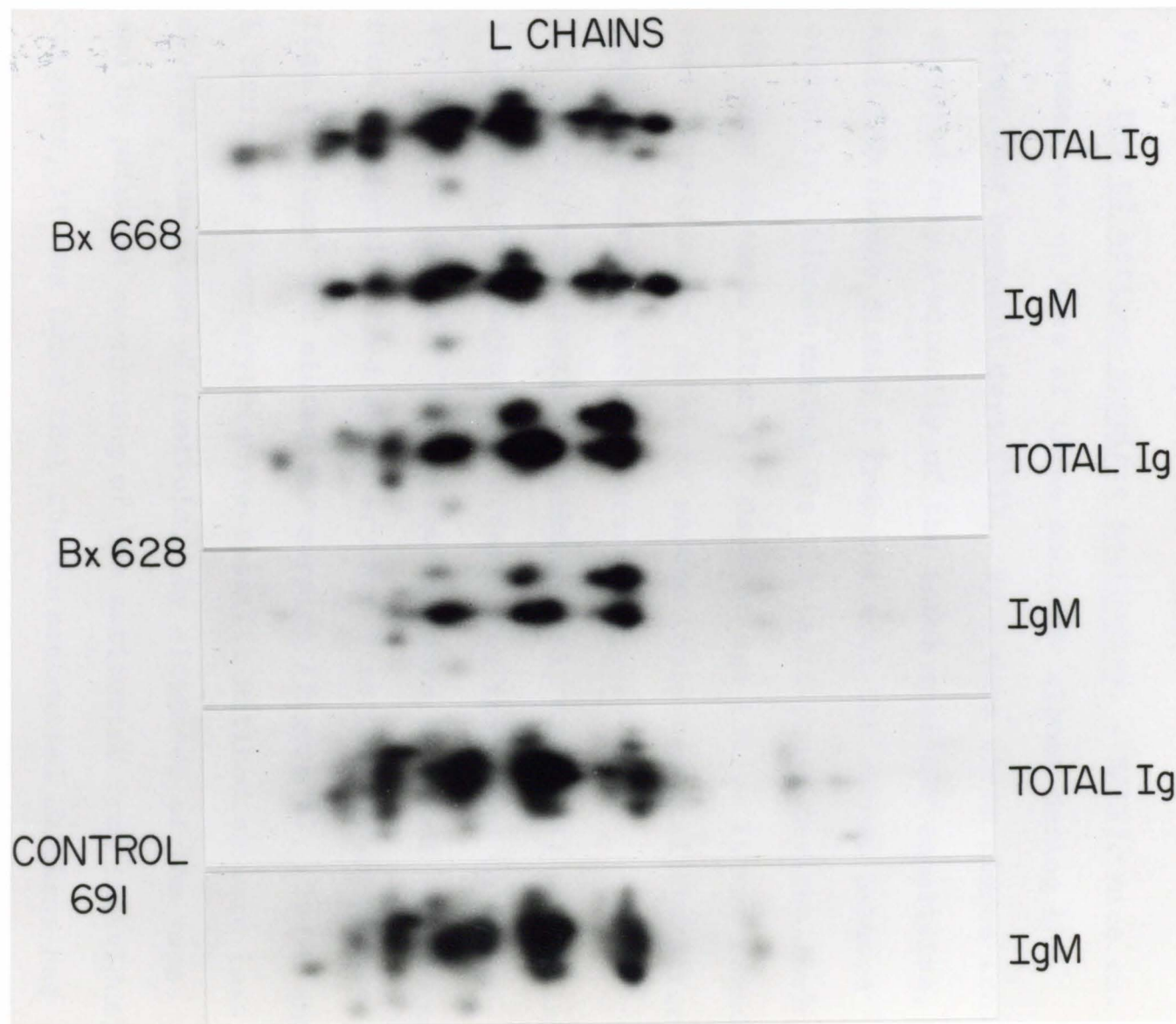
between the μ chain spots of bursectomized chickens and controls, the IgM L chain spots of bursectomized chickens again showed a large range in diversity (Table 1), showing that the limited L chain diversity was present in both IgG and IgM.

In both control and bursectomized chickens the L chain spots of IgM and of total immunoglobulins were very similar (figure 5). The differences observed can be attributed to the lower sensitivity of the IgM maps: a one-week exposure of the IgM map gave L chain spots virtually identical to a 60-hour exposure of the total-immunoglobulin map. This shows that similar sets of L chain spots were associated with both μ and γ chains.

IV. Stability of Limited Ig Diversity. - A bursectomized chicken that showed no immunoglobulin spots at 9 weeks of age still had no immunoglobulin spots at 27 months. So agammaglobulinemia is stable. Three different chickens which showed limited diversities of immunoglobulin spots at 12 weeks of age gave almost identical immunoglobulin spots when assayed at 22, 31, and 32 weeks of age respectively. The difference between the two patterns from each chicken was mostly a change of intensity of some of the L chain spots. These spots may either increase or decrease in

Figure 5. Comparison of L chains from immunoprecipitated total immunoglobulins and from immunoprecipitated IgM on two-dimensional autoradiographic (60-hour exposure) maps. The IgM maps were less sensitive than the total immunoglobulin maps and fainter spots in the latter were not detectable in the former. These faint spots became detectable with a one-week exposure of the X-ray films. The amplification obtained was 2-5x over the 60-hour exposures.

L CHAINS



intensity. In two chickens 2-3 L chain spots in each had disappeared; no new spots were found.

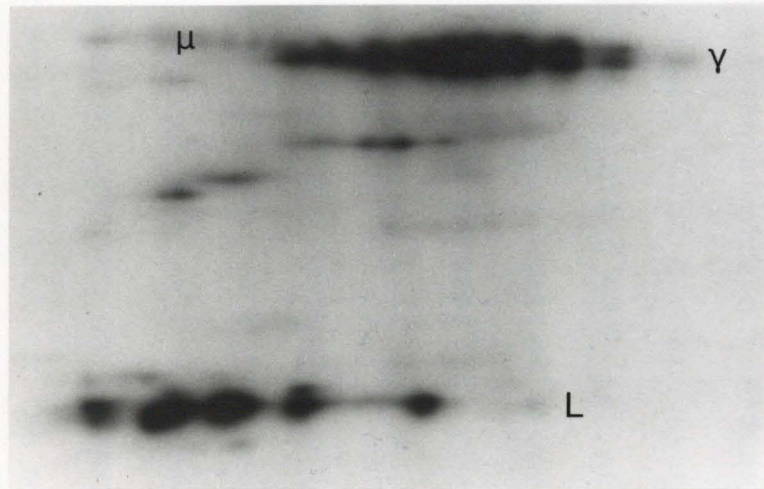
V. Map of antigen-specific antibodies. - BALB/c mice expressed one or more of three anti-DNP clones during the first four neonatal days (35). By 9 days these clones constituted only a minority of the total anti-DNP repertoire. Anti-TNP clones distinct from the anti-DNP clones behaved similarly. Clones making the T15 (anti-phosphorylcholine) idiotype appeared after 6-7 days of age (36). It was found that bursectomized chickens showed these neonatal characteristics of limited antibody diversity.

Three bursectomized chickens (673, 628, 648) with relatively high immunoglobulin diversities responded to sheep erythrocyte ghosts. The antibodies were isolated by adsorption to lightly-fixed sheep erythrocytes (antigenic properties retained) and eluted for mapping (figure 6). The H and L chains of sheep erythrocyte-specific antibodies were less diverse than those of controls. By alignment of the maps and by pairwise co-running of the antibodies from individual chickens, it was found that the bursectomized chickens had very similar L chain spots. At least one spot was present in all three chickens and 4 spots each appeared in two chickens. There were 6 unique spots, 4 appearing in the

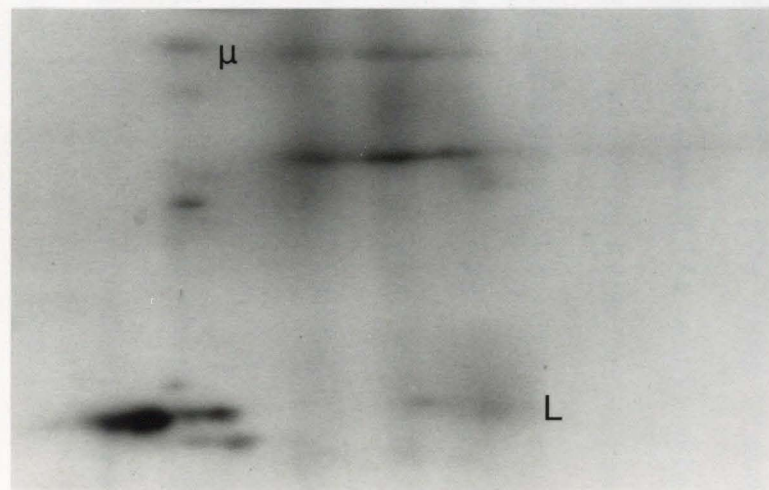
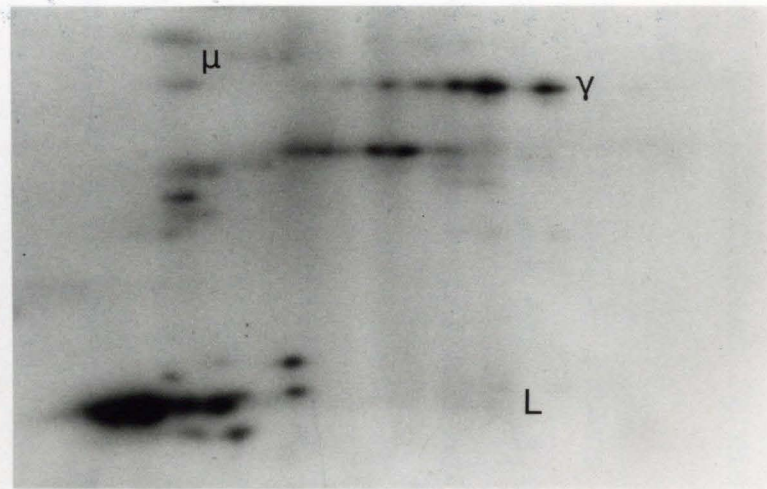
Figure 6. Maps of anti-sheep erythrocyte antibodies from a control bird (691) and three bursectomized responders (673, 628, 648). μ , γ , L indicate immunoglobulin spot positions. Chicken 673 did not make any IgG antibodies against sheep erythrocytes. In a map of a non-responder only the non-immunoglobulin spots were seen; three spots, due to protein X, were present in the L chain area. Analysis in text takes account of this observation.

ANTI-SHEEP ERYTHROCYTE Ig

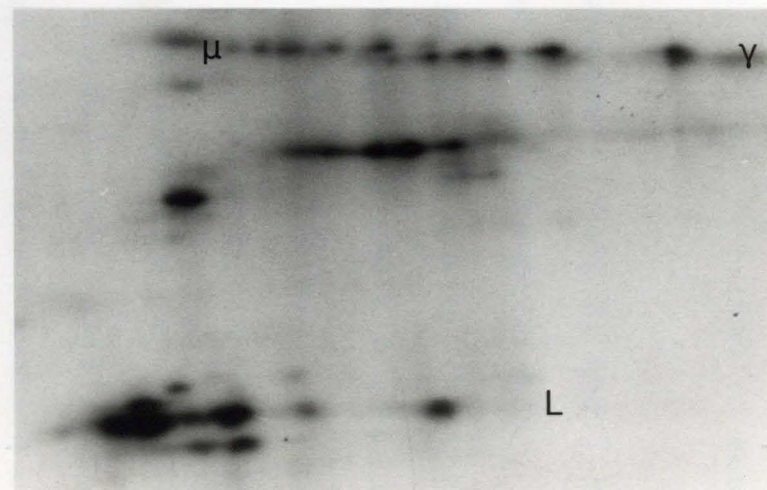
CONTROL 69I



Bx 628



Bx 673



Bx 648

chicken with the highest Ig diversity, and one each in the other two.

Chicken 673 had 5 μ spots compared to 4-5, 8, and 7-8 μ spots for chickens 628, 648, and control respectively (Table 3). Chicken 673 had no γ spots. By co-running the two samples the 7-8 γ spots of chickens 628 and 648 showed an identical pI spectrum, but the spots of chicken 648 had slightly lower mobilities in the SDS dimension. Each of the eleven control γ spots of the control also were resolved into 2-3 "subspots" with identical pI but different mobilities. The difference in mobility may reflect different numbers of uncharged carbohydrate residues attached to the same γ chain.

Similarly the L chain spots of DNP-specific antibodies of chickens 628 and 648, isolated by elution from DNP-lysine-Sepharose, showed limited diversity compared to control spots, and were extensively shared. Chicken 628 did not have any γ chain spots; otherwise the same μ and γ spots were seen in both bursectomized and control chickens. Co-running of the anti-DNP antibodies of chickens 628 and 648 on two-dimensional gels confirmed the extensive sharing of the L chains apparent on comparison of the individual maps. No new spots were seen in the mixed sample, and the same number of total spots were found from the two individual

Table 3

	Sheep erythrocyte-specific*			DNP-specific*		
Chicken						
	μ	γ	L	μ	γ	L
Bx 673 ⁺	5	0	3	--- [‡]	---	---
Bx 628	4-5	7-8	5	8-9	0	15
Bx 648	8	7-8	9	8-9	9-10	17
691 [§]	7-8	11	10	9	9-10	26

Table 3. Antigen-specific antibodies of bursectomized chickens.

* Antibodies were adsorbed to the appropriate immunoabsorbent, eluted, and mapped. Spots corresponding to these found with non-responders were considered as non-immunoglobulin.

⁺ Bx, bursectomized chicken.

[‡] Chicken 673 did not respond to DNP.

[§] Normal, control chicken.

maps and from the mixing map.

The observed patterns of antigen-specific antibodies were not due to non-specific binding to the immunoabsorbents. First, the immunoglobulin spots were enriched over the other serum components. Second, the L chain spots seen in these maps constitute only a subset of the total immunoglobulin L chain spots of each chicken. Finally, chicken 673 had no γ chains in its DNP-specific antibodies and chicken 628 had no sheep erythrocyte-specific γ chains, although both had γ chains demonstrable by the maps of total serum immunoglobulins. It is not clear why these chickens made only IgM and not IgG antibodies.

VI. Diversity of Immunoglobulins and Antigen-specific Antibodies

The diversity of antigen-specific antibody spots paralleled that of total immunoglobulin or IgM spots. The major diversity difference observed between bursectomized chickens and controls was in the L chains and γ chains, with little or no difference seen in the μ chains. The diversity of the antigen-specific antibody spots of individual chickens was also correlated with their total immunoglobulin diversities. Of all the chickens bursectomized on day 11, chicken 648 had the most diverse L chains, indistinguishable from those of controls in the map of total serum immunoglobulins. With a larger immunoglobulin diversity the potential repertoire is

larger, and more antigen-specific clones are available to expand out in response to antigen. In addition the mapping of antigen-specific antibodies of chicken 648 showed that although the resolution of the mapping procedure was inadequate to distinguish between the diversity of its immunoglobulin L chains and those of controls, its low titer response to antigen and less diverse antigen-specific antibodies showed that it too had limited immunoglobulin diversity.

Discussion

We have presented direct evidence for stable limited immunoglobulin diversity in chickens hormonally bursectomized on day 11 of incubation. The degree of limited diversity showed the greatest range in the L chains; the γ chains were less affected; while little or no difference was seen in the μ chains. For a given bursectomized chicken its IgM and IgG were associated with the same set of limited diversity L chains. Antigen-specific antibodies made by bursectomized chickens in response to 6 weekly immunizations were of limited diversity and their diversity was correlated with the total immunoglobulin diversity of each responding chicken.

Previous studies of day 3-, day 13- (37), and day 14-

bursectomized chickens (38) failed to detect limited immunoglobulin diversity using urea-starch gel and urea-acrylamide gel electrophoresis respectively. The difference in results obtained can be attributed to the relative resolving power of the techniques used. We find that day 18-, and even a day 11-bursectomized chicken judged to have limited immunoglobulin diversity by its lower titer response to antigen and by the limited diversity of its antigen-specific antibodies, had immunoglobulin patterns indistinguishable from those of controls in the two-dimensional maps. With isoelectric focussing alone, IgM purified by Na_2SO_4 precipitation of sera from bursectomized chickens showed minor unconvincing differences (Huang and Dreyer, unpublished observations): since μ and L chains had similar pI's (5.7 - 7.6 and 5.5 - 7.5 respectively) it was difficult to detect low diversity L chains against a background of μ chain bands of normal diversity. It appears that the critical elements are the complexity of the immunoglobulins in the sera, and the resolving power of the technique. If the immunoglobulins are of relatively high diversity the individual chains are not resolved even by using a high-resolution method. Instead, analysis of e.g. antigen-specific antibodies must be used. If the separation procedure is of relatively low resolution

then even relatively low diversity immunoglobulins would not be resolved into discrete species, away from other serum components.

It is possible that the present procedures may not detect high diversity immunoglobulins which are present in the bursectomized chickens at very low levels. If high diversity immunoglobulins exist they are present in very low concentrations. The total concentration for such high diversity immunoglobulins must be $<30 \mu\text{g/ml}$ (sensitivity limit of the mapping procedure). If there are only 100 such clones of B cells, each clone cannot contribute more than $0.3 \mu\text{g/ml}$, or it would be detected. The limited immunoglobulin diversity of bursectomized chickens is stable. An agammaglobulinemic chicken had no immunoglobulins when assayed at 9 weeks and at 27 months of age. Chickens with limited immunoglobulin diversities showed a stable pattern over at least 20 weeks. Although in two cases L chain spots were lost, there was no evidence of new spots. If there was a high diversity of B-cell clones in these animals it would be expected that, in response to environmental antigens, clonal expansion of at least some of the clones would occur and their products would become detectable. This was not observed. When the chickens were subjected to repeated

antigenic challenge the few with the highest immunoglobulin diversity did respond. In the responders the antigen-specific antibody spots were of limited diversity, resembling the condition seen in neonatal mice (34, 35). In addition there was extensive sharing of the antigen-specific spots between the different responders. It is unlikely that in animals with normal clonal diversities identical clones are expanded out in response to multiple challenge with sheep erythrocytes or DNP. For example, in genetically identical Xenopus heterogeneity of anti-sheep erythrocyte clonotypes were observed on repeated challenge (39). In the same study it was also found that previous rejection of a skin graft, before immunization with DNP-KLH introduced differences in the anti-DNP clonotypes of genetically identical individuals. In the absence of previous antigenic challenge such frogs made almost identical anti-DNP and anti-SE clonotypes in a primary response. The bursectomized chickens responded to multiple antigenic challenge with identical, limited numbers of clones because these were the only clones available in their repertoire. It was found that the different chickens showed extensive sharing of their μ , γ and L chains (Huang and Dreyer, in preparation). Again it is unlikely that such extensive sharing can result if clonal

expansion in response to environmental antigens was from a large repertoire. The conclusion is that bursectomized chickens have a genuinely limited collection of B-cell clones, and this limited diversity restricts the diversity of immunoglobulins they can make, as well as in their ability to respond to antigenic challenge.

It is important to note that a normal serum-immunoglobulin concentration does not necessarily imply that a normal immunoglobulin diversity exists (12). Bursectomized chickens may have normal immunoglobulin levels or may even be hypergammaglobulinemic, yet they still had poor antibody responses to a variety of antigens (6-8, 12, 17). These observations are consistent with the bursectomized chickens having up to normal or supernormal quantities of limited diversity immunoglobulins, made by relatively few clones of B lymphocytes (8), and therefore having limited ability to respond to antigenic challenge.

The finding of limited-immunoglobulin diversity in bursectomized chickens shows that the normal generation of diversity had been blocked. The establishment of limited diversity occurred in ovo as hormonal bursectomy on day 18 of incubation resulted in immunoglobulin patterns indistinguishable from those of controls. The great increase in

diversity between day 11 and day 18 was enough to overwhelm the resolution of the two-dimensional mapping method.

Chickens bursectomized on day 11 of incubation and examined at 12 weeks of age showed a progression of stable limited diversity in their γ and L chains; and only the birds with the highest L chain diversities responded to antigenic challenge. Among these the diversity of the antigen-specific antibodies was correlated with the total immunoglobulin diversity. This suggests that in some bursectomized chickens the generation of diversity had progressed farther than in others before being blocked. It is possible that some human hypogammaglobulinemia may result from a defect in the generation of diversity (29, 40).

The precise mechanism by which testosterone blocks the generation of diversity is unknown. It has been shown that after 3-5 days' exposure to testosterone neither the stem cell population nor the bursal tissues were irreversibly damaged (3), although on prolonged exposure the lymphoid buds and follicles degenerated into cystic cavities, and the bursal tissue showed fibrous changes and epithelial proliferation (5). Houssaint et al. (2) suggest that only close contact with bursal epithelial cells allowed stem cells to differentiate into lymphocytes, otherwise granulopoiesis occurred. The epithelium overlying the follicles

is indeed functionally and morphologically distinguishable from the interfollicular epithelium (41, 42). Bursopoietin isolated from bursae can induce bone marrow cells to differentiate and express Bu-1, a chicken B cell-surface antigen (43), analogous to the thymopoietin induction of prothymocytes to express distinctive thymocyte surface antigens (44). It is plausible that testosterone may act by interfering with the normal induction of B stem cells by the bursal environment.

Whatever the mechanism involved in the blockage of the generation of diversity it seems that the surviving clones found in bursectomized chickens are those which had matured beyond the testosterone-sensitive stage. It is unknown whether such cells had already seeded to the periphery (10) or if they still resided in the bursa and subsequently escaped before bursal degeneration. It would be informative to assay directly the immunoglobulin diversity in embryonic bursae of different ages.

The characteristics of the 'surviving' immunoglobulins in chickens bursectomized on day 11 of incubation may be summarized as follows. The L chains showed the greatest range of limited diversity, followed by γ chains, while μ chains appeared normal. Without knowing the size of the normal

repertoire of V_H and V_L genes it is not possible to decide if one family is expressed earlier than the other. It could be that the V_H repertoire is small, in which case a larger fraction of the V_H repertoire would be expressed at any given time compared to the more diverse V_L repertoire. The effect of bursectomy would then appear to be greatest on the L chains even though all the families are expressed at the same rate. It is therefore necessary to determine the V_L and V_H repertoire sizes in normal chickens. This may be accomplished by increasing the resolution of the H and L regions of the two-dimensional gels. Individual spots may then be eluted to check if each is indeed a single polypeptide chain.

In any bursectomized chicken the L chains of IgM and total immunoglobulins appear to be very similar (figure 5). This suggests that any L chain may associate with either a μ or a γ chain. It is consistent with B cells switching from expression of one C_H region gene to another (14, 15, 45) but retaining the same V_H , V_L and C_L gene expression (46).

A range of stable and limited γ and L chain diversities was found in the bursectomized chickens. A few chickens of relatively high L chain diversity responded to antigens.

The diversity of antigen-specific L chains was correlated with the total immunoglobulin L chain diversity. These observations suggest that the developmental generation of immunoglobulin diversity was blocked at slightly different points in different chickens. Therefore it is possible to infer the developmental strategies used in the generation of diversity by analysis of the particular H and L chains made by each chicken. One can also study the generation of diversity by reconstituting lethally irradiated mice with fetal liver cells (47). The advantage of the chicken system is that larger amounts of materials are available. Each bleeding may yield 15 ml sera, and since the spot patterns are stable, pooled sera may be used for the isolation of the polypeptides in the spots. Stocks of sera accumulated from weekly bleedings (over 350 ml in one case) will be invaluable for the isolation and amino-acid sequence studies of individual immunoglobulin chains. The studies will be useful for determining the relationship between immunoglobulins made by normal and bursectomized chickens.

Finally, the bursectomized chickens showed extensive similarities in their antigen-specific L chains, and in their serum immunoglobulin H and L chains (Huang and Dreyer, in preparation). Detailed analyses indicate that if chick-

ens have a reasonably large (200-300) V_L gene repertoire then it is likely that an identical orderly sequence of V gene expression exists in different individuals.

Summary

Immunoglobulins made by chickens bursectomized in ovo on day 11 of incubation were studied by two-dimensional gel electrophoresis. It was found the bursectomized chickens have limited immunoglobulin diversity. The degree of limited diversity in individual chickens showed the greatest range in the light chains, the γ chains were less affected, while μ chains appeared normal. The limited immunoglobulin diversity was stable for at least 20 weeks in that the same spots were found over this time span. Bursectomized chickens which responded to repeated antigenic challenge had sheep erythrocyte- and dinitrophenol-specific antibodies of limited diversity. Different bursectomized responders made almost identical antigen-specific antibodies. The results were interpreted as indicating that different bursectomized birds were blocked at different points in the developmental generation of immunoglobulin diversity.

REFERENCES

1. Romanoff, A. L., 1969. The Avian Embryo. Structural and Functional Development. The Macmillan Co., New York, p. 505.
2. Houssaint, E., M. Belo, and N. M. LeDouarin. 1976. Investigation on cell lineage and tissue interactions in the developing bursa of Fabricius through inter-specific chimeras. Dev. Biol. 53:250.
3. Moore, M. A. S., and J. J. T. Owen, 1966. Experimental studies on the development of the bursa of Fabricius. Dev. Biol. 14:40.
4. Edwards, J. L., R. C. Murphy, and Y. Cho. 1975. On the development of the lymphoid follicles of the bursa of Fabricius. Anat. Rec. 181:735.
5. Warner, N. L. and F. M. Burnet, 1961. The influence of testosterone treatment on the development of the bursa of Fabricius in the chick embryo. Aust. J. Biol. Sci. 14:580.
6. Cooper, M. D., R. D. A. Peterson, M. A. South, and R. A. Good. 1966. The functions of the thymus system and the bursa system in the chicken. J. Exp. Med. 123:75.
7. Warner, N. L., J. W. Uhr, J. Thorbecke, and Z. Ovary. 1969. Immunoglobulins, antibodies and the bursa of Fabricius: induction of agammaglobulinemia and the loss of all antibody-forming capacity by hormonal bursectomy. J. Immunol. 103:1317.
8. Kincade, P. W., K. S. Self, and M. D. Cooper. 1973. Survival and function of bursa-derived cells in bursectomized chickens. Cell. Immunol. 8:93.
9. Lydyard, P. M., C. R. Grossi, and M. D. Cooper, 1976. Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. J. Exp. Med. 144:79.
10. Grossi, C. E., P. M. Lydyard, and M. D. Cooper, 1976.

B-cell ontogeny in the chicken. Ann. Immunol. (Inst. Pasteur) 127C:931.

11. Glick, B., T. S. Chang, and R. A. Jaap. 1956. The bursa of Fabricius and antibody production. Poult. Sci. 35:224.
12. Van Meter, R., R. A. Good, and M. D. Cooper. 1969. Ontogeny of circulating immunoglobulins in normal, bursectomized and irradiated chickens. J. Immunol. 102:370.
13. Cooper, M. D., W. A. Cain, P. J. Van Alten, and R. A. Good. 1969. Development and function of the immunoglobulin producing system. I. Effect of bursectomy at different stages of development on germinal centers, plasma cells, immunoglobulins and antibody production. Int. Arch. Allergy Appl. Immunol. 35:242.
14. Kincade, P. W., A. R. Lawton, D. E. Bockman, and M. D. Cooper. 1970. Suppression of Immunoglobulin G synthesis as a result of antibody-mediated suppression of Immunoglobulin M synthesis in chickens. Proc. Nat. Acad. Sci. U.S.A. 67:1918.
15. Leslie, G. A. and L. N. Martin. 1973. Modulation of immunoglobulin ontogeny in the chicken: effect of purified antibody specific for μ chain on IgM, IgY, and IgA production. J. Immunol. 110:959.
16. Ivanyi, J. 1975. Immunodeficiency in the chicken. I. Disparity in suppression of antibody responses to various antigens following surgical bursectomy. Immunology 28:1007.
17. Pierce, A. E., R. C. Chubb, and P. L. Long. 1967. The significance of the bursa of Fabricius in relation to the synthesis of 7s and 19s immune globulins and specific antibody activity in the fowl. Immunology 10:321.
18. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007.
19. Hunter, W. M. and F. C. Greenwood. 1962. Preparation

- of Iodine -131 labelled human growth hormone of high specific activity. Nature (Lond.) 194:495.
20. Morrow, P. R. 1974. Associated effects of blood group loci in chickens. M. S. Thesis. University of California, Davis.
 21. Leslie, G. A. and L. W. Clem. 1969. Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken. J. Exp. Med. 130:1337.
 22. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. J. Biol. Chem. 245:3059.
 23. Choi, Y. S. and R. A. Good. 1972. Development of chicken lymphoid system. I. Synthesis and secretion of immunoglobulins by chicken lymphoid cells. J. Exp. Med. 135:1133.
 24. Neal, N. W. and J. R. Florini. 1973. A rapid method for desalting small volumes of solution. Analyt. Biochem. 55:328.
 25. Huang, H. V., R. S. Molday, and W. J. Dreyer. 1973. Isoelectric focussing of rod outer segment membrane proteins. FEBS Lett. 37:285.
 26. Fairbanks, A., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem. 10:2606.
 27. Scheidegger, J. J. 1955. Une micro-methode d'immuno-electrophorese. Int. Arch. Allergy Appl. Immunol. 7:103.
 28. Vaerman, J.-P., A. M. Legacq-Verheyden, L. Scolari, and J. F. Heremans. 1969. Further studies on radial immunodiffusion. I. Direct proportionality between area of precipitate and reciprocal of antibody concentration. Immunochem. 6:279.
 29. Hong, R., and R. A. Good. 1967. Limited heterogeneity of gammaglobulin in hypogammaglobulinemia. Science 156:1102.

30. Kubo, R. T., I. Y. Rosenblum, and A. A. Benedict. 1970. The unblocked N-terminal sequence of chicken IgG λ -like light chains. J. Immunol. 105:533.
31. Kubo, R. T., I. Y. Rosenblum, and A. A. Benedict. 1971. Amino terminal sequence of heavy and light chains of chicken anti-dinitrophenol antibody. J. Immunol. 107:1781.
32. Grant, J. A., B. Sanders, and L. Hood. 1971. Partial amino acid-sequences of chicken and turkey immunoglobulin light chains. Homology with mammalian λ chains. Biochemistry 10:3123.
33. Dayhoff, M. O. 1972. Atlas of Protein Sequence and Structure 1972. The National Biomedical Research Foundation. Washington, D. C.
34. Cullen, S. E. and B. D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens using immunoprecipitation induced by Protein A - bearing *Staphylococci*. J. Immunol. 117:136.
35. Klinman, N. R. and J. L. Press. 1975. The characterization of B-cell repertoire specific for the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal BALB/c mice. J. Exp. Med. 141:1133.
36. Sigal, N. H., P. J. Gearhart, J. L. Press, and N. R. Klinman. 1976. Late acquisition of a germ line antibody specificity. Nature (Lond.) 259:51.
37. Gold, E. F. and A. A. Benedict. 1967. Comparison of polypeptide chains of G-globulin from bursectomized and normal chickens. Proc. Soc. Exptl. Biol. Med. 125:535.
38. Leslie, G. A. 1975. Polyclonal nature of the IgM from dysgammaglobulinemic chickens. Immunol. Comm. 4:529.
39. Wabl, M. R. and L. DuPasquier. 1976. Antibody pattern in genetically identical frogs. Nature (Lond.) 264:642.
40. Lawton, A. R., L. Y. F. Wu, and M. D. Cooper. 1973. A spectrum of B-cell differentiation defects. In "Immuno-

deficiency in Man and Animals". D. Bergsma, editor. Sinauer Assoc., Inc., Sunderland, 28.

41. Bockman, D. E. and M. D. Cooper. 1973. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. Am. J. Anat. 136:455.
42. Holbrook, K. A., W. D. Perkins, and B. Glick. 1974. The fine structure of the bursa of Fabricius: "B" cell surface configuration and lymphoepithelial organization as revealed by scanning and transmission electron microscopy. J. Reticuloendothel. Soc. 16:300.
43. Brand, A., D. A. Gilmar, and A. Goldstein. 1976. Lymphocyte-differentiating hormone of bursa of Fabricius. Science 193:319.
44. Basch, R. S. and A. Goldstein. 1974. Induction of T-cell differentiation in vitro by Thymin, a purified polypeptide hormone of the thymus. Proc. Soc. Nat. Acad. Sci. U.S.A. 71:1474.
45. Bleux, C., M. Ventura, and P. Liacopolous. 1977. IgM-IgG switch-over among antibody-forming cells in the mouse. Nature (Lond.) 267:709.
46. Fair, D. S., C. Sledge, R. G. Krueger, K. G. Mann, and L. E. Hood. 1975. Studies on IgA and IgG monoclonal proteins derived from a single patient. Evidence for identical light chains and variable regions of the heavy chain. Biochemistry 14:5566.
47. Rozing, J., N. H. C. Brons, and R. Benner. 1977. B lymphocyte differentiation in lethally irradiated and reconstituted mice. II. Recovery of humoral immune responsiveness. Cell. Immunol. 29:37.

CHAPTER II

Orderly Readout of Antibody Variable Region Genes in Chickens

Abstract

Bursectomized chickens are blocked in their generation of immunoglobulin diversity and have restricted diversity immunoglobulins and antigen-specific antibodies. A range of different degrees of diversity restriction was found in chickens bursectomized on day 11 of incubation, presumably due to the blockage of the generation of diversity at slightly different stages in different chickens. Analysis by two-dimensional gels showed that very similar antigen-specific antibody and serum immunoglobulin heavy and light chains were found in individual chickens. Statistical calculations were made to test if the extensive similarities could arise from random gene expression during development. The results indicate that if chickens have a normal repertoire of 200-300 V-region genes coding for immunoglobulin L chains then it is unlikely that random gene expression could result in such extensive similarities. The implications of an orderly readout of immunoglobulin V genes are discussed.

Introduction

Immunoglobulins are composed of heavy (H) and light (L) polypeptide subunits (Gally, 1973). Each polypeptide subunit consists of an N-terminal variable (V) and a C-

terminal constant (C) region (Gally and Edelman, 1972). These regions are coded by separate genes (Hood, 1972; Hozumi and Tonegawa, 1976; Tonegawa, et al., 1977). In mammals two L chain multigene families, κ and λ , and one heavy chain family, H, code for the immunoglobulin subunits (Mage, et al., 1973; Hood, 1973). In a family there are multiple V-region genes and one or a few constant region genes (Gally and Edelman, 1972). Thus commitment to expression of a particular H or L chain involves at least two not necessarily separate events. First, particular V-region and C-region genes are selected. Second, a V-C joining event brings the separate genes together, resulting in a continuous VC gene together with its regulatory region (Tonegawa, et al., 1977). The gene can now be expressed by the cell. Previous studies suggested that the heavy chain constant region (C_H) genes are ontogenetically expressed in the order μ then γ . (Kincade, et al., 1970; Lawton, et al., 1972; Bleux, et al., 1977). The order of expression of other C_H genes is yet unclear. In this paper we ask if there is an orderly expression of variable region genes of H chains (V_H) and L chains (V_L) during development.

It was shown that hormonal bursectomy in ovo blocks the generation of immunoglobulin diversity (Huang and Dreyer, in

preparation). Bursectomized chickens have limited IgG and IgM diversity. This was reflected in their deficient and limited-diversity antibody responses to antigenic challenge. The limited immunoglobulin diversity persisted over at least 20 weeks; except for quantitative changes the same H and L chain pattern was found over this time span. The bursectomized chickens showed a range of diversity limitation, presumably due to the blockage of the generation of immunoglobulin diversity at slightly different times in different chickens. It is therefore possible to reconstruct the developmental process of V_H and V_L expression by analyzing with two-dimensional gel electrophoresis the H and L chains made by each bursectomized chicken. The analysis presented here shows the extensive similarities of the immunoglobulins made by individual bursectomized chickens, and suggests that if chickens have 200-300 V_L genes then it is likely that there is an orderly expression of immunoglobulin V genes.

Materials and Methods

The bursectomy of chickens and the analysis of their immunoglobulins by two-dimensional gel electrophoresis were as previously described (Huang and Dreyer, in preparation). Briefly, chicken embryos were hormonally bursectomized by injection of testosterone propionate dissolved in corn oil

on day 11 of incubation (Warner, et al., 1969). Controls were treated identically except that the corn oil did not contain testosterone. Chickens were immunized by 6 weekly injections with dinitrophenyl (DNP)-bovine gamma globulins, sheep erythrocytes, and human hemoglobin. Immunization was initiated at 6 weeks of age and the chickens were bled at 12 weeks of age, one week after the last immunization. Sera were stored under liquid nitrogen until use. Sera were iodinated to very high specific activities (ca. 1 mCi ^{125}I ml $^{-1}$ serum) (Hunter and Greenwood, 1962). The immunoglobulins were purified by immunoabsorption to insolubilized antigen or immunoprecipitation with rabbit anti-chicken immunoglobulins followed by goat anti-rabbit immunoglobulins. The immunoglobulins were reduced, denatured, and the H and L chains were separated by isoelectric focussing followed by electrophoresis in a sodium dodecylsulfate (SDS) exponential-gradient gel (O'Farrell, 1975). The H and L chains were detected by autoradiography.

Exposed spots on the autoradiographs were photoenlarged ca. 1.5X, and traced onto tracing paper. The spots from each of sixteen bursectomized and four normal control chickens were compared with spots from all the other chickens. Reproducibility of electrophoretic spots was excellent ex-

cept in the alkaline region ($\text{pH} > 7.6$) and allowed direct superimposition of the autoradiographs or the tracings for spot identification. Spots were considered identical only if they had identical pI 's and SDS gel mobilities.

Immunoglobulin spots were identified as those corresponding to spots on a gel of immunoglobulins purified from pooled sera (Leslie and Clem, 1969). Furthermore, chicken sera was passed repeatedly over a column of rabbit anti-chicken immunoglobulins coupled to Sepharose 4B (Cuatrecasas, 1970). This immunoglobulin-depleted sera was subjected to the same immunoprecipitation and gel electrophoresis as the sera from the individual chickens. All spots corresponding to those found on the autoradiograph of the gel of immunoglobulin-depleted sera were considered as non-immunoglobulin.

Some non-immunoglobulin polypeptides were found to co-purify with immunoglobulins when immunoprecipitation was achieved with goat anti-rabbit immunoglobulins. They were entirely eliminated if the second antibody was substituted with formalin-fixed, protein A-bearing staphylococci (Cullen and Schwartz, 1976). The non-immunoglobulin spots were useful nonetheless. They did not interfere with the immunoglobulin spots, and served as landmarks for the correct

alignment of the autoradiographs and tracings.

Seven chickens with relatively high L chain diversities had large, intense L chain spots overlying spots found in other chickens. The intense spots were considered to be composites of the latter. This can potentially lead to over-estimation of the L chain diversity of these chickens. In the data analysis of L chains this possibility was taken into account (see Discussion). This problem was not encountered with the H chains or with chickens with relatively low L chain diversity.

Results

Antigen Specific Antibodies

Two and three bursectomized chickens with the highest immunoglobulin diversities responded to immunization with DNP and sheep erythrocytes respectively. The L chains of the antibodies made by these chickens in response to multiple immunizations with sheep erythrocytes and with DNP were of limited diversity (figure 1). The diversity of the antibodies made by a chicken was correlated with the diversity of its total serum immunoglobulins, in that a chicken which had a higher total immunoglobulin diversity also had a higher diversity of antigen-specific H and L chains. Different individuals made very similar antigen-specific L chain spots

Figure 1. L chain spots of anti-DNP antibodies from a control bird (691) and two bursectomized responders (628, 648). Aliquots of 250,000 cpm, either samples from each bird, or a 125,000 cpm: 125,000 cpm mixture of birds 628 and 648 respectively, were applied to the gels. The spots shared between these chickens is graphically shown in figure 2B. Bx, bursectomized chicken.

ANTI-DNP Ig, L CHAINS



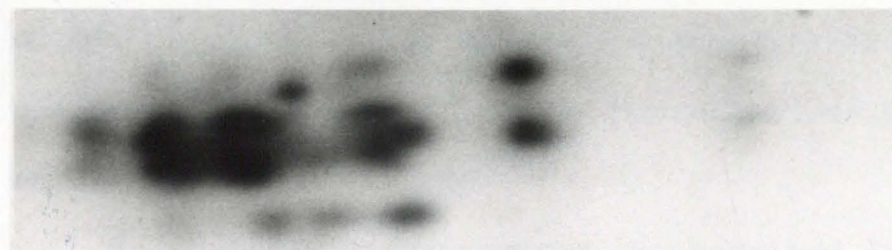
CONTROL
691



Bx
628



Bx
648



Bx 628
+
Bx 648

(figure 1, 2). This similarity was confirmed by corunning on the two-dimensional gels mixed samples from pairs of chickens. The same number of total spots was found by counting the spots on the autoradiograph of the mixed sample, or by comparison of the autoradiographs of the individual samples. Thus antigen-specific antibody spots are shared by different chickens.

Serum Immunoglobulins

The analysis of the antigen-specific antibodies suggested that bursectomized chickens should have very similar serum immunoglobulins. Examination of the serum immunoglobulins confirmed this suggestion.

A.) Heavy chain spots. The same set of μ spots appeared in autoradiographs of purified serum IgM of all the chickens examined, both bursectomized and normal. The differences found were that bursectomized chickens had fainter spots and the relative intensities of the μ spots were unlike that of normal chickens. This suggests a quantitative but not a qualitative difference between bursectomized and normal chickens.

Most of the bursectomized chickens had fainter and less diverse γ spots compared to controls (figure 3). A few γ spots in the alkaline region ($pI > 7.6$) could not be com-

Figure 2. Graphical representation of the sharing of antigen-specific L chain spots. Spots were identified from autoradiographs of two-dimensional gels of purified antigen-specific antibodies. Background spots found in a non-responder were excluded in the analysis of spot sharing. A. Sheep-erythrocyte-specific L chains (autoradiographs not shown). Chickens 673, 628 and 648 are three bursectomized chickens, chicken 691 is a normal control. These birds had 3, 5, 9 and 10 anti-sheep erythrocyte L chain spots respectively. B. DNP-specific L chains (autoradiographs shown in figure 1). Chickens 628 and 648 are two bursectomized chickens, chicken 691 is a normal control. These birds had 15, 17 and 26 anti-DNP L chain spots respectively. The numbering of the spots is arbitrary.

A.

76

B.

SPOT
NO.

10 —

5 —

1 —

673

628

648

691

SPOT
NO.

30 —

25 —

20 —

15 —

10 —

5 —

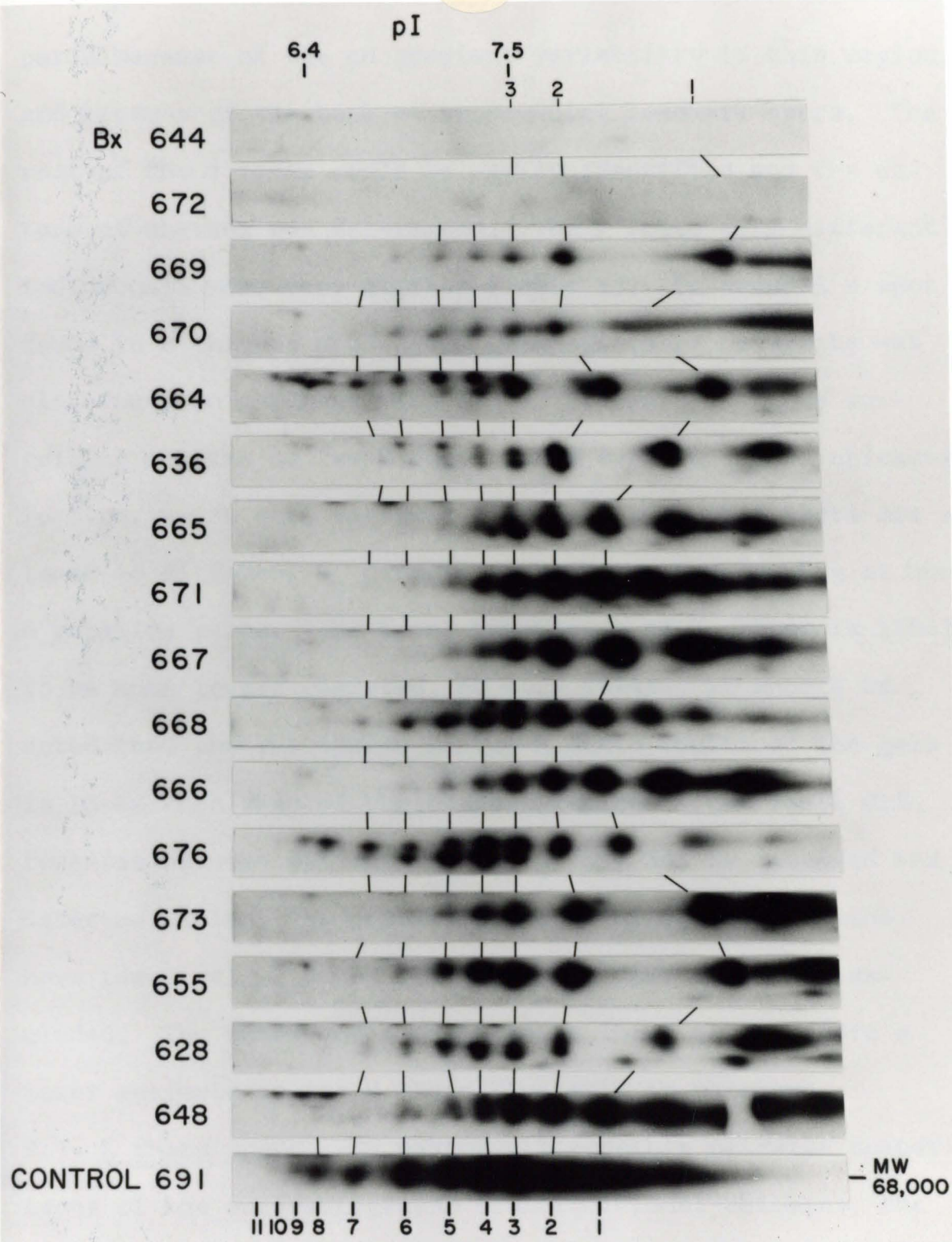
1 —

628

648

691

Figure 3. γ chain spots of serum immunoglobulins. The order from top to bottom of the figure conforms to an increasing L chain spot diversity. Bx, bursectomized chicken. These autoradiographs were used for the analysis of γ chain spot sharing (Table 1). Autoradiographs of purified IgM (not shown) were used for the analysis of μ chain spot sharing. Some spots were too faint to be photographed. Different chickens had identical spots 1-7, indicated by vertical lines connecting the spots. The occurrence of spots 8-11 is shown only for the control chicken (691). The identity of spot 1 is tentative because of the pH gradient variability in this region. Spots more alkaline than spot 1 could not be compared.



pared because of the pH gradient variability in this region, and because of the lack of surrounding landmark spots. The rest of the γ spots could be easily identified and the extent of sharing was determined. The γ spots from different individuals were very similar (Table 1). In general a spot found in a chicken with lower immunoglobulin diversity was also found in chickens with higher diversity. It is surprising to find so few H chain spots even in normal chickens. In mice, amino acid sequence studies showed that there are at least 16 different V_H protein sequences, representing at least 8 germline genes. The total repertoire of V_H genes is likely to be much larger (Barstad, et al., 1974). It should be noted that the resolution in the H chain region of the gels is lower than that of the L chain region. Thus small differences between different H chains may not be resolved and detected. Also, the possibility that different H chains have identical pI's and SDS-gel mobilities cannot be excluded. The number of H chain spots found is therefore a lower estimate of the H chain diversity in chickens.

B.) L chain spots. By making all possible pairwise comparisons of the autoradiographs from individual chickens, the number of L chain spots in each chicken was determined. A wide range of L chain diversity was found (figure 4). In-

Table 1
Occurrence of Chain Spots

Chicken	Spot Number*												Alkaline Spots†	Total Spots
	1	2	3	4	5	6	7	8	9	10	11	12		
Bx § 644	+	+	+										0-1	3-4
672	+	+	+	+									2	6
669	+	+	+	+	+	+	+						2	9
670	+	+	+	+	+	+	+						3	10
664	+	+	+	+	+	+	+	+	+				2	11
636	+	+	+	+	+	+	+						2	9
665	+	+	+	+	+	+	+						2-3	9-10
671	+	+	+	+	+	+	+		+				4-5	12-13
667	+	+	+	+	+	+	+						1-2	8-9
668	+	+	+	+	+	+	+	+					5	13
666	+	+	+	+	+	+	+						2-3	9-10
676	+	+	+	+	+	+	+	+	+	+			3	12
673	+	+	+	+	+	+	+		+				1-2	9-10
655	+	+	+	+	+	+	+	+	+	+			0-2	9-11
628	+	+	+	+	+	+	+						2-3	9-10
648	+	+	+	+	+	+	+	+					4	12

08

Chicken	Spot Number*												Alkaline Spots	Total Spots
	1	2	3	4	5	6	7	8	9	10	11	12		
Control														
691	+	+	+	+	+	+	+	+	+	+	+		2	13
692	+	+	+	+	+	+	+	+	+	+	+	+	2	14
694	+	+	+	+	+	+	+	+	+	+	+	+	4	17

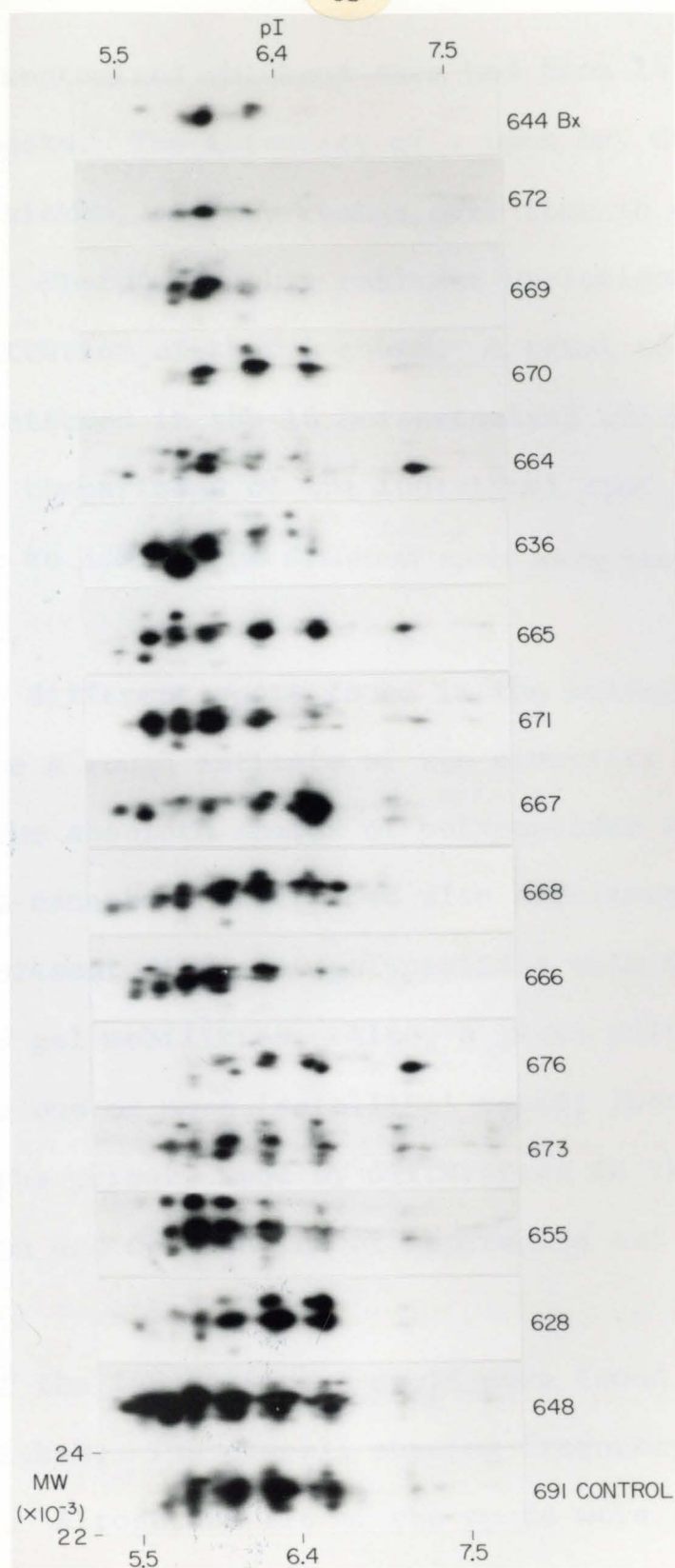
* Spots were identified by making all pairwise comparisons of the autoradiographs of gels of serum immunoglobulin. The same arbitrary system was used in this table and in figure 3 to number the γ spots.

181

† Spots in the alkaline (pH > 7.6) region could not be identified with certainty.

§ Bx: bursectomized chicken.

Figure 4. L chain spots of serum immunoglobulins. Bursectomized chickens had from 14 to 83 L chain spots each, as determined by the pairwise comparisons of the autoradiographs of the gels of purified serum immunoglobulins. The order from the top to the bottom of the figure is the same as in figure 3, and conforms to an increasing L chain spot diversity. The normal control (691) had too many spots to be resolved by the two-dimensional gel technique. Some of the faint spots are lost in the reproduction process. Note that the sensitivity was better for detection of L chains of bursectomized chickens since identical amounts of radioactivity (250,000 cpm) were used in all the gels, while the amount of protein used was much less for the gels of bursectomized chickens (e.g., chicken 648 had 24% of control levels of immunoglobulins). A longer exposure (1 week instead of 60 hours) of the gel of control chicken 691 showed that its L chain pattern was indistinguishable from that of chicken 648.



individual bursectomized chickens each had from 14 to at least 83 L chain spots. The intensity of a spot may differ from chicken to chicken, and may change over time in an individual chicken. Presumably this reflects variations in the serum concentration of that L chain. A total of 696 L chain spots were detected in the 16 bursectomized chickens. From the pairwise comparisons of the individual spot patterns it was possible to identify 168 different spots among the total of 696 (Figure 5).

The 168 different spots found in the bursectomized chickens give a rough estimate of the diversity of chicken L chains. The absolute number of polypeptides represented by the spots cannot be determined with certainty since a spot may represent different polypeptides with identical pI's and SDS gel mobilities. Also, a given polypeptide may give rise to one or more 'satellite' spots; these may be derived from the primary spot by differences in the extent of glycosylation and deamidation of asparagine and glutamine residues.

Most of the 168 different spots were found in more than one chicken. The overall sharing frequency is shown in figure 6. Altogether 126 of the spots were shared. These represent 94% of the 696 total spots found. In any

Figure 5. Graphical representation of the particular L chain spots found in individual bursectomized chickens. Spots were identified from pairwise comparisons of the autoradiographs shown in figure 4. The spots are arbitrarily numbered 1 through 168. A solid bar indicates the presence of the spot in the particular chicken. For instance, spot 1 was found in all the chickens, while spot 150 was found only in chicken 655.

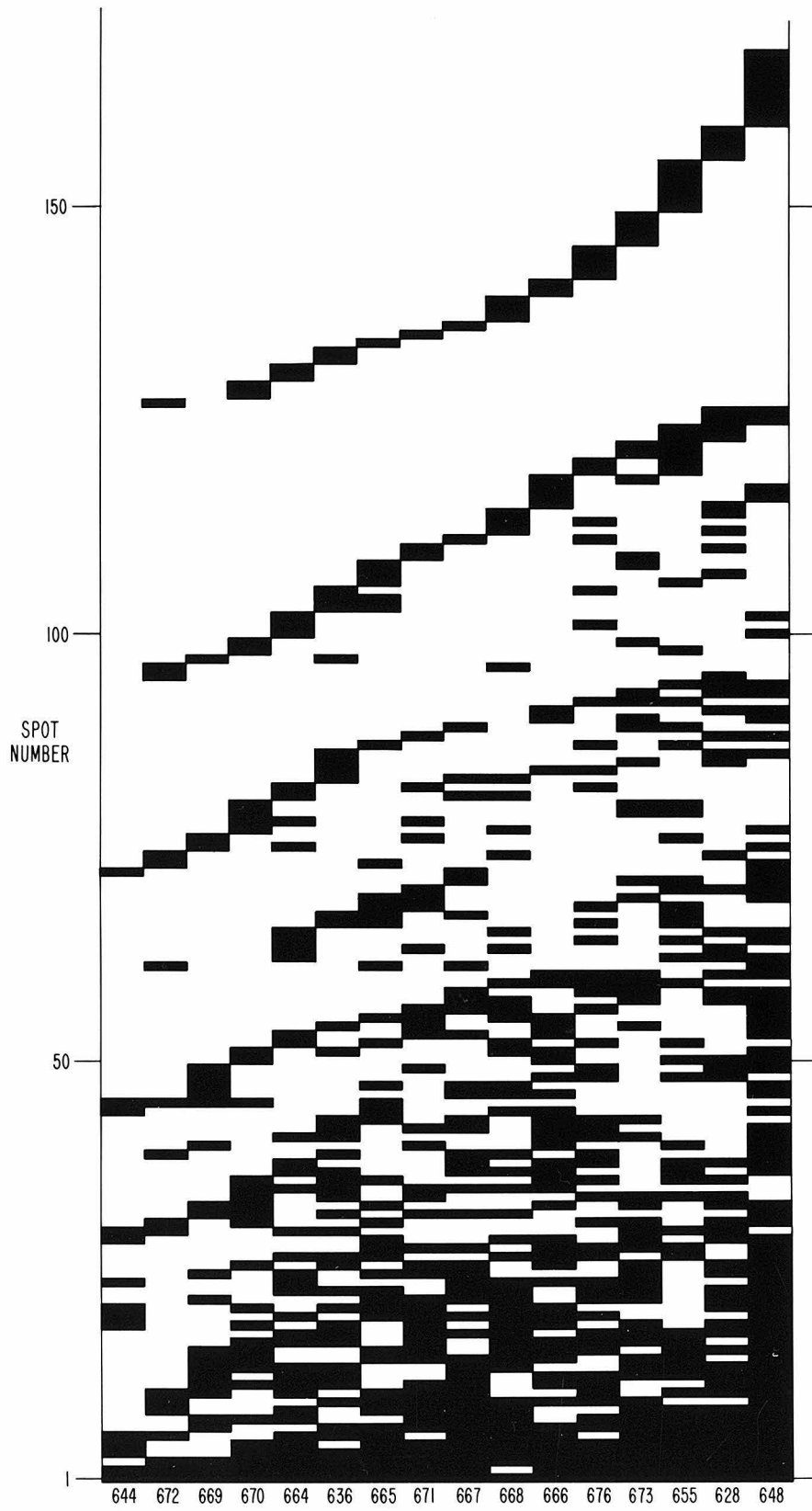
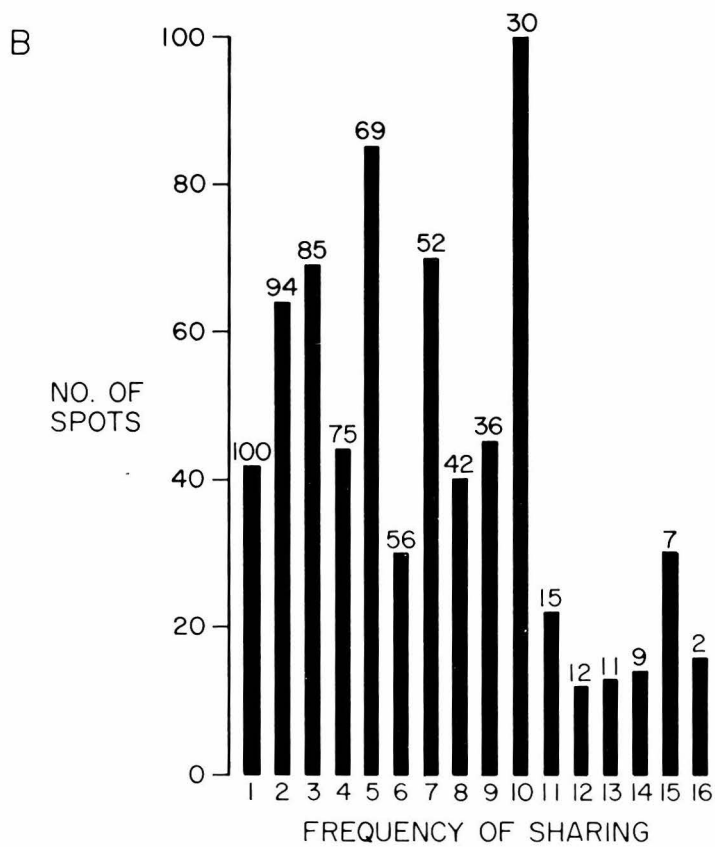
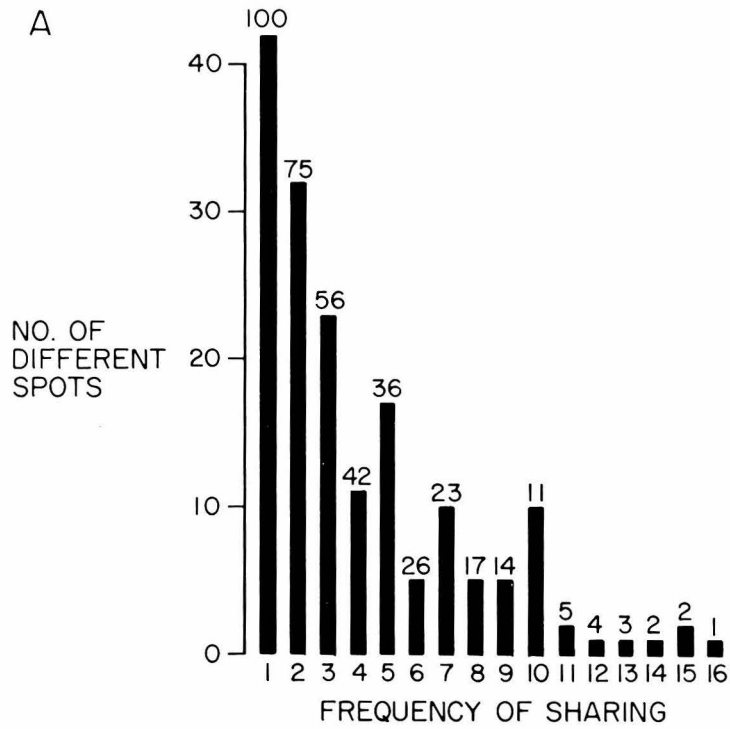


Figure 6. Sharing of L chain spots. A. Distribution of spots into sharing-frequency classes. For example, of the 168 different spots identified from the pairwise comparisons, ten of them were each found in 10 different bursectomized chickens. Eleven percent of the 168 different spots were each found in ten or more chickens. B. The total number of spots falling into each sharing-frequency class. For instance, of the 696 spots found in the 16 bursectomized chickens, 100 of them fall into the 10X sharing class. Thirty percent of the 696 spots fall into the 10X or higher sharing classes.



pairwise comparisons an average of 50% (range 21-86%) of the spots from the chicken with a lower L chain diversity was found in the chicken with a higher L chain diversity (figure 7). Thus extensive similarities of L chain spots were found in individual bursectomized chickens.

Discussion

The two-dimensional gel electrophoresis gives excellent resolution of polypeptides with small differences in their pI's and their SDS-gel mobilities (O'Farrell, 1975). However, the possibility that two different polypeptides have identical pI's and SDS-gel mobilities cannot be excluded. For example, if one polypeptide differs from the other by the replacement of a leucine residue by an isoleucine residue, the polypeptides would probably not be resolved. They would co-migrate through both dimensions and give a single spot. Thus in the subsequent discussion when spot identity is taken to imply polypeptide identity, it is subject to this qualification.

Extensive immunoglobulin H and L chain similarities were found between individual bursectomized chickens. Whether this extensive similarity is fortuitous or not depends on the size of the normal V gene repertoire. If the repertoire is large such that each bursectomized chicken ex-

Figure 7. The number of L chain spots of serum immunoglobulins shared between any two bursectomized chickens. The extent of spot sharing was determined by making all pairwise comparisons between the L chain spots of individual bursectomized chickens. Only a minimum estimate of the number of L chain spots could be made for chicken 648. The sharing of L chain spots between chicken 648 and the other chickens was not included in the statistical calculations.

CHICKEN	644	14																	
	672	5	17																
	669	3	5	24															
	670	6	5	12	32														
	664	8	7	10	11	39													
	636	7	7	9	15	16	41												
	665	10	11	12	12	13	16	42											
	671	7	6	15	18	18	17	19	43										
	667	8	9	15	12	22	21	20	23	45									
	668	10	8	11	15	20	19	20	21	26	48								
	666	11	5	12	18	15	23	20	14	22	23	49							
	676	6	7	12	12	21	21	21	22	24	20	24	52						
	673	9	8	11	17	17	20	20	21	25	20	24	23	53					
	655	6	7	13	17	20	17	20	19	25	17	18	27	25	56				
	628	9	10	14	17	20	18	18	22	20	21	21	21	26	24	58			
	648	12	11	20	21	29	25	28	33	37	34	39	30	34	27	39	83		
		644	672	669	670	664	636	665	671	667	668	666	676	673	655	628	648		
		CHICKEN																	

Number of spots shared
between chickens

presses only a relatively small subset of it, then random gene expression would be unlikely to result in extensive sharing of the immunoglobulins.

The sharing of H chain spots was consistent with an orderly V_H gene commitment (Table 1). However, there were relatively few H chain spots. If this was taken to imply that there are few V_H genes, and that most of the genes are expressed in any given bursectomized chicken, then it is possible that random gene expression could account for the extensive sharing.

For the apparently much more diverse L chains it becomes less likely that the extensive sharing could result from random expression of V_L genes. Statistical calculations were made on the sharing data obtained from autoradiographs of gels of purified total immunoglobulins and purified IgM (figure 7). The question asked was how many V_L genes must there be such that a random expression of genes would be unlikely to lead to the observed degree of sharing.

Suppose the total repertoire size is T . A random expression of V genes in the bursectomized chickens may be modeled by randomly picking N_1 genes from T to correspond to a chicken with N_1 spots. Similarly, N_2 genes are picked from T for another chicken with N_2 spots. The probability that in

comparing the N_1 and N_2 genes there are S or more genes in common is given by the hypergeometric distribution. Calculations were made for all the pairwise comparisons except those involving the bursectomized chicken with the highest immunoglobulin diversity:

$$\text{Probability that sharing is } \geq S = 1 - \sum_{S_i=0}^{S-1} \frac{\binom{N_1}{S_i} \binom{T-N_1}{N_2-S_i}}{\binom{T}{N_2}}$$

$$\text{where } \binom{A}{B} = \frac{A!}{B!(A-B)!};$$

and N_1, N_2 = the number of spots in chickens 1 and 2 respectively; S = the observed number of spots shared; and T = total number of V_L genes.

With about 200 total V_L genes the probability that the observed sharing is due to random gene expression falls below 0.01 (Table 2). Similar calculations were made for the comparisons between the four chickens with the least L chain diversity. This was to test the possibility that the sharing may be overestimated in comparing chickens with relatively high diversities. These chickens had 14, 17, 24, and 32 L chain spots respectively (figures 5, 7). Again relatively few V_L genes were required. Thus at a 99% confidence level the observed sharing is due to an orderly expression

Table 2

V_L Gene Repertoire Size Required for Random Readout to be
Unlikely

L Chain Sharing of	Chickens Compared	Mean Number of V_L Genes at P 0.01 ($\pm$ S.D.)*
Total Ig	all [†]	193 (86)
IgM	all [†]	233 (321)
Total Ig	4 least diverse [§]	304 (215)
IgM	4 least diverse [§]	119 (62)

Calculations were made using data shown in figure 7 for the sharing of L chains of serum immunoglobulins (Total Ig).

Similar calculations were made using data from gels of purified IgM (not shown) for the sharing of L chains of serum IgM.

* Mean number of V_L genes required for P 0.01 that the observed sharing is due to random V gene readout; S.D., standard deviation.

† All chickens compared except for the chicken with the highest Ig diversity (chicken 648).

§ Comparisons of four chickens with the least Ig diversity (chickens 644, 672, 669, 670).

of V_L genes if chickens have a repertoire of 200-300 V_L genes.

The size of the V_L gene repertoire is unknown. In mice amino-acid sequence and DNA hybridization studies suggest that there are one or a very few V_λ and a minimum of 35 V_κ germline genes (Cohn, et al., 1974; Tonegawa, et al., 1977). The estimated total number of germline V_L genes range from ca. 100 to thousands of genes (Cohn, et al., 1974; Hood, et al., 1974). It is possible that somatic mutations may further increase the V_L repertoire by some unknown amount (Tonegawa, et al., 1974; Cohn, et al., 1974). We found a total of 168 different spots in the 16 bursectomized chickens. This is a conservative estimate of the potential V_L repertoire, since these chickens have limited immunoglobulin diversities and do not express the total possible repertoire (Huang and Dreyer, in preparation). Hence it is plausible that chickens have a V_L repertoire of at least 200-300 different V_L genes, in which case the orderly expression of V_L genes is likely.

Ideally an orderly expression of V genes should result in total sharing of the L chains. If the chicken with the least diversity of L chains had L chains 1 to 14, then the next chicken should have L chains 1 to 14 plus extra ones;

and the next chicken yet should have all L chains found in the second chicken, as well as further extra ones, and so on. However, under experimental conditions the variability seen in the spots made by the bursectomized chickens (figure 5) is not unexpected. Once the commitment was made to express a particular V_L gene the lymphocyte must be seeded from the bursa to the periphery, and there to proliferate and produce detectable amounts of serum immunoglobulins. It seems likely that this may account for some of the variability. The analogous variability in the appearance of particular DNP-responsive clones and the relative clone frequencies in neonatal mice may similarly reflect variations in the size of specific clones in the neonatal mouse (Klinman and Press, 1975). Thus the variability may arise from the dynamics of cell seeding and cell proliferation, and need not contradict an orderly V_L gene expression.

The strongest evidence for an orderly V_L gene expression is the extensive sharing of the L chains of antigen-specific antibodies (figure 2). The antigen-specific antibody L chains of bursectomized chickens are of limited diversity compared to those of normal chickens, and constitute a subset of the limited diversity L chains made by bursectomized chickens. Furthermore, the antigen-specific

L chains are extensively shared. If V_L gene expression was random, then different bursectomized chickens would each express a random subset of the total repertoire. The likelihood is low that these chickens would respond to antigens with similar L chains. An orderly V_L gene expression results in extensive sharing. Individual bursectomized chickens, having identical and orderly programs for V_L gene expression, are blocked in the generation of Ig diversity at slightly different stages. The chickens would have very similar limited diversity L chain repertoires, and therefore similar antigen-specific L chains, also of limited diversity.

X-ray crystallographic studies of antibodies showed that amino acid residues of the V regions of both H and L chains lie in the antigen-binding pocket (Davies, et al., 1975). Antigen binding occurs by non-covalent bonding between the antigen and the side chains of these residues. An orderly expression of V genes generates B cells with specific cell-surface antibodies in an orderly fashion. The binding of antigen to the cell-surface antibodies is the primary step in a humoral immune response (Raff, 1973). Thus an orderly appearance of the functional capabilities of the humoral immune system would be expected if V gene

expression is orderly. Indeed, in chicken embryos the bursal B cells which bind keyhole limpet hemocyanin or a synthetic polypeptide (poly-L-(Tyr, Glu)-poly-D,L-Ala-poly-Lys) appear earlier than cells which bind sheep erythrocytes (Lydyard, et al., 1976).

Once antigen is bound the B cell may proliferate and differentiate to produce large amounts of serum antibodies. Alternately the cell may be tolerized; that is, become unresponsive to antigenic challenge (Nossal and Pike, 1975; Weigle, 1973). The complicating factor is that these responses may require the participation of accessory immune cells such as T cells (Cantor, et al., 1975). Previous studies which found an orderly appearance of responsiveness to different antigens in newly hatched chicks (Ivanyi, 1975) and in the fetal sheep (Silverstein and Prendergast, 1969) did not take into account the requirement for T cells. Thus it is not clear if B cells were the limiting factor. Perhaps the appropriate B cells were present, but mature T cells were not, thus resulting in non-responsiveness until the latter cells became competent.

This complication was removed in experiments where lethally irradiated mice were used for in vivo tissue culture (Klinman, 1972). The antigen responsiveness of neo-

natal B cells were tested in the presence of competent T cells. It was found that within an inbred strain there was a sequential and orderly appearance of clones of B cells making antibodies specific for different antigens (Press and Klinman, 1974; Sigal, et al., 1976; Klinman and Press, 1975). The same clones appeared at the same time in different individuals. Furthermore, the induction of tolerance by antigen was easiest when clones responsive to the antigen first appear (Metcalf et al., 1977). Thus the functional capabilities of the humoral immune system do appear in an orderly fashion, and imply that this is due to an orderly expression of V genes.

An orderly expression of V genes is an efficient strategy to read out the genetic information contained in the antibody multigene families. If there are 300 V genes in an antibody multigene family, then 300 orderly 'readout events' will totally express this genetic information. In contrast, random V-gene expression is not as efficient. As the random V-gene expression proceeds the probability increases that some previously chosen gene is picked again. Computer simulations of a random expression of V-genes from a repertoire of 300 genes showed that to express 90% (270 genes) of the repertoire, an average of 701 (range, 580-858) readout

events are required (Huang and Dreyer, unpublished results).

The orderliness of V gene expression does not indicate how the V genes are actually read out. Are the readouts from different antibody multigene families coordinated? In any B cell the expression of paternal versus maternal genes is mutually exclusive (Cebra, et al., 1966). The mechanism for this allelic exclusion is unknown. In any B cell the expression of κ or λ genes is also mutually exclusive (MÅke-lå and Cross, 1970). A cell line was derived from the fusion of two human B cell lymphomas. One of the parental lymphomas expressed κ L chains and the other expressed λ L chains. The hybrid cells expressed both κ and λ chains at the appropriate parental levels (Rosen, et al., 1977). This implies that each L chain family is under autonomous control in the hybrid cells, and that trans-repression of gene expression in one family by the other family is insignificant. Thus the coordination in determining which L chain family is to be utilized is likely to occur at the time of L chain commitment. Whether there is coordination of H and L families is yet to be established.

In the expression of V genes, are contiguous V genes read out in a sequence one after another? If antibody multigene families evolve by rapid expansion and contraction

through homologous but unequal crossovers it is likely that adjacent genes are more closely related than genes far from each other (Black and Gibson, 1974; Hood, 1973; Smith, 1976). Closely related genes code for polypeptides with similar amino acid sequences. The amino acid sequence studies of antigen-binding myelomas have shown that antibody subunits of similar sequences have similar antigen binding properties (Hood, et al., 1975). This clustering of similar V genes plus an orderly V gene readout may explain how in neonatal mice there is an almost simultaneous appearance of multiple different cell clones responsive to the same antigen (Klinman and Press, 1975).

In a given family can the V gene readout start at multiple origins? This question arose from the consideration that a strictly orderly readout starting at one origin is slow. For example, assuming a cell cycle time of 10 hours and that each cell division reads out one gene, it would take 125 days to complete the readout of 300 genes. If there are 10 readout origins it would take only 10-20 days. In this model the primordial B lymphocyte precursor gives rise to stem-cell daughters each committed to one readout origin. The particular readout origin may differ from stem cell to stem cell. The multigene family is therefore par-

tioned into sublibraries. Subsequently, perhaps triggered by signals from the appropriate inductive environment, an orderly V gene readout may then proceed. Thus we hypothesize the existence of a genetic mechanism which determines the readout origins, and partitions the repertoire among the stem cells. The stem cells then give rise to lymphocytes each committed to a V gene in the sublibrary. It is likely that if multiple readout origins and sublibraries exist they can impart very sophisticated genetic and evolutionary capabilities to the antibody multigene families (Strosberg, 1976).

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REFERENCES

- Barstad, P., Farnsworth, V., Weigert, M., Cohn, M., and Hood, L. (1974). Mouse immunoglobulin heavy chains are coded by multiple germ line variable region genes. *Proc. Nat. Acad. Sci. USA* 71, 4096-4100.
- Black, J. A., and Gibson, D. (1974). Neutral evolution and immunoglobulin diversity. *Nature (London)* 250, 327-328.
- Bleux, C., Ventura, M., and Liacopolous, P. (1977). IgM-IgG switch-over among antibody-forming cells in the mouse. *Nature (London)* 267, 709-711.
- Cantor, H., Shen, F. W., and Boyse, E. A. (1975). Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen: after immunization antigen specific suppressor and helper activities are mediated by distinct T-cell subclasses. *J. Exp. Med.* 143, 1391-1401.
- Cebra, J. J., Coleberg, J., and Dray, S. (1966). Rabbit lymphoid cells differentiated with respect to α -, γ -, and μ heavy polypeptide chains and to allotypic markers Aa1 and Aa2. *J. Exp. Med.* 123, 547-558.
- Cohn, M., Blomberg, B., Geckeler, W., Raschke, W., Riblet, R., and Weigert, M. (1974). First order considerations in analyzing the generator of diversity. In "The Immune System. Genes, Receptors, Signals" (E. E. Sercarz, A. R. Williamson, and C. F. Fox, eds.), 89-117. Academic Press, New York.
- Cuatrecasas, P. (1970). Protein purification by affinity chromatography. *J. Biol. Chem* 245:3059-3065.
- Cullen, S. E., and Schwartz, B. D. (1976). An improved method for isolation of H-2 and Ia alloantigens using immunoprecipitation induced by Protein A-bearing *Staphylococci*. *J. Immunol.* 117:136-142.
- Davies, D. R., Padlan, E. A., and Segal, D. M. (1975). Three dimensional structure of immunoglobulins. *Ann. Rev. Genet.* 44, 639-667.

- Gally, J. (1973). Structure of Immunoglobulins. In "The Antigens" (M. Sela, ed.), Vol. I, 162-299. Academic Press, New York.
- Gally, J., and Edelman, G. M. (1972). The genetic control of immunoglobulin synthesis. *Ann. Rev. Genet.* 6, 1-46.
- Hood, L. (1972). Two genes: one polypeptide -- fact or fiction? *Fed. Proc.* 31, 177-187.
- Hood, L. (1973). The genetics, evolution and expression of antibody molecules. *Stadler Genet. Symp.* 5, 73-142.
- Hood, L., Barstad, P., Loh, E., and Nottenburg, C. (1974). Antibody diversity: an assessment. In "The Immune System. Genes, Receptors, Signals" (E. E. Sercarz, A. R. Williamson, C. F. Fox, eds.), 119-139. Academic Press, New York.
- Hood, L., Campbell, T. H., and Elgin, S. C. R. (1975). The organization, expression, and evolution of antibody genes and other multigene families. *Ann. Rev. Genet.* 9, 305-353.
- Hozumi, N., and Tonegawa, S. (1976). Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc. Nat. Acad. Sci. USA* 73, 3628-3632.
- Hunter, W. M. and Greenwood, F. C. (1962). Preparation of Iodine -131 labelled human growth hormone of high specific activity. *Nature (London)* 194:495-496.
- Ivanyi, J. (1975). Immunodeficiency in the chicken. I. Disparity in suppression of antibody responses to various antigens following surgical bursectomy. *Immunology* 28; 1007-1013.
- Kincade, P. W., Lawton, A. R., Bockman, D. E. and Cooper, M. D. (1970). Suppression of Immunoglobulin G synthesis as a result of antibody-mediated suppression of Immunoglobulin M synthesis in chickens. *Proc. Nat. Acad. Sci. USA* 67, 1918-1925.
- Klinman, N. R. (1972). The mechanism of antigenic stimula-

tion of primary and secondary clonal precursor cells. J. Exp. Med. 136, 241-260.

Klinman, N. R., and Press, J. L. (1975). The characterization of B-cell repertoire specific for the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal BALB/c mice. J. Exp. Med. 141;1133-1146.

Lawton, A. R., Asofsky, R., Hylton, M. B., and Cooper, M. D. (1972). Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ -chain. J. Exp. Med. 135, 277-297.

Leslie, G. A., and Clem, L. W. (1969). Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken. J. Exp. Med. 130;1337-1352.

Lydyard, P. M., Grossi, C. R., and Cooper, M. D. (1976). Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. J. Exp. Med. 144;79-97.

Mage, R., Lieberman, R., Potter, M., Terry, W. D. (1973). Immunoglobulin allotypes. In "The Antigens" (M. Sela, ed.), Vol. 1, 300-377, Academic Press, New York.

Mäkelä, O., and Cross, A. M. (1970). The diversity and specialization of immunocytes. Prog. Allergy 14, 145-207.

Metcalf, E. S., Sigal, N. H., Klinman, N. R. (1977). In vitro tolerance induction of neonatal murine B cells as a probe for the study of B-cell diversification. J. Exp. Med. 145, 1382-1386.

Nossal, G. J. V., and Pike, B. L. (1975). Evidence for the clonal abortion theory of B-lymphocyte tolerance. J. Exp. Med. 141, 904-917,

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250; 4007-4021.

Press, J. L., and Klinman, N. R. (1974). Frequency of hapten-specific B cells in neonatal and adult murine spleens. Eur. J. Immunol. 4, 155-159.

- Raff, M. (1973). T and B lymphocytes and immune responses. *Nature* (London) 242, 19-23.
- Rosen, A., Clements, G., Klein, G., and Zenthen, J. (1977). Double immunoglobulin production in cloned somatic cell hybrids between two human lymphoid cell lines. *Cell* 11 139-147.
- Sigal, N. H., Gearhart, P. J., Press, J. L., and Klinman, N. R. (1976). Late acquisition of a germ line antibody specificity. *Nature* (London) 259; 51-52.
- Smith, G. P. (1976). Evolution of repeated DNA sequences by unequal crossover. *Science* 191, 528-535.
- Silverstein, A. M., and Prendergast, R. A. (1969). Lymphogenesis, immunogenesis and the generation of immunologic diversity. In "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and H. Riah, eds.), 69-77, Academic Press, New York.
- Strosberg, A. D. (1976). A possible control by regulatory allelic genes of allotype expression. *Biochem. Soc. Trans.* 4, 41-44.
- Tonegawa, S., Hozumi, N., Matthysens, G., and Schuller, R. (1977). Somatic changes in the content and context of immunoglobulin genes. *Cold Spring Harbor Symp. Quant. Biol.* 41, 877-890.
- Tonegawa, S., Steinberg, C., Duke, S., and Bernadini, A. (1974). Evidence for somatic generation of antibody diversity. *Proc. Nat. Acad. Sci. USA* 71, 4027-4031.
- Warner, N. L., Uhr, J. W., Thorbecke, J., and Ovary, Z. (1969). Immunoglobulins, antibodies and the bursa of Fabricius: induction of agammaglobulinemia and the loss of all antibody-forming capacity by hormonal bursectomy. *J. Immunol.* 103; 1317-1330.
- Weigle, W. O. (1973). Immunological unresponsiveness. *Adv. Immunol.* 16, 61-122.

Future Prospects

The results and conclusions in chapters I and II are based on a major assumption. The assumption is that the humoral immune system of 12 week old chickens bursectomized in ovo is identical to that when the generation of diversity was blocked. Although it was shown that the limited immunoglobulin diversity was stable over at least 20 weeks, changes in the diversity before 12 weeks of age cannot be excluded. Another uncertainty is the precise effects of testosterone treatment on B cell development. It would be necessary to test the immunoglobulin diversity of normal embryos of various ages. To do this would require solving some technical problems. It will be necessary to detect very small amounts of embryonic immunoglobulins against a background of large quantities of maternal immunoglobulins (1). One approach is to use in vivo incorporation of radiolabeled amino acids to specifically label the embryonic immunoglobulins. Another approach is to establish an in vitro organ culture of the bursal matrix (2) and then to introduce blood-borne B stem cells and induce them to develop in the culture. This approach has the potential for the identification and isolation of single stem cells. A further possibility is to transform the stem cells to obtain permanent, homogeneous cell lines

(3). If successful this approach may allow a step by step dissection of the ontogenetic expression of variable-region genes.

The relationship between immunoglobulins made early in development may be studied by isolating individual L chains made by the bursectomized chickens. Large pools of sera were collected for this purpose. Since chicken L chains are very similar in their N-terminal amino acid sequences (4) it is necessary for sequence studies to sequence through at least the first hypervariable region to establish good genealogic trees. Even with large pools of sera the amount of individual L chains obtainable is likely to be very small and micro-sequencing techniques may be necessary. These sequence studies are also useful in determining if a single spot on the gels really represents a single polypeptide. If such is the case the use of two-dimensional gels, perhaps with improved resolution, will greatly expedite the isolation of individual H and L chains.

Parallel studies should be conducted on mammals. The transplantation of fetal liver cells into lethally irradiated mice is one possible system (5). Strategies similar to those described for the chick embryo systems may similarly be applied to the mammalian system.

In summary, the generation of immunoglobulin diversity is an excellent model system for the study of the expression of genes in complex multigene families. Besides the basic question of the developmental program used by a given multigene family, one could also investigate the interactions and coordination between different multigene families. The especially intriguing question is, what role does DNA modification play in these developmental events?

REFERENCES

1. Rose, M. E., E. Orlans, and N. Buttress: Immunoglobulin classes in the hen's egg: their segregation in yolk and white. Eur. J. Immunol. 4:521-523 (1974).
2. Elde, R., W. Werner, D. Johnson, E. J. Kennedy, and A. Bartha: Immunological function study of the bursa of Fabricius: Immunocompetence inducing agent. Trans. Illinois Acad. Sci. 63:64-66 (1970).
3. Galfre, A., S. C. Howe, C. Milstein, A. W. Butcher, and J. C. Howard: Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature (London) 266:550-552 (1976).
4. Grant, J. A., B. Sanders, L. Hood: Partial amino acid sequences of chicken and turkey immunoglobulin light chains. Homology with mammalian λ chains. Biochemistry 10L3123-3132 (1971).
5. Rozing, J., N. H. C. Brons, and R. Benner: B lymphocyte differentiation in lethally irradiated and reconstituted mice. II. Recovery of humoral immune responsiveness. Cell. Immunol. 29:37-53 (1977).

CHAPTER III

Application of Latex Microspheres in the Isolation of Plasma
Membranes.

Affinity Density Perturbation of Erythrocyte Membranes

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APPLICATION OF LATEX MICROSPHERES IN THE ISOLATION OF PLASMA MEMBRANES

AFFINITY DENSITY PERTURBATION OF ERYTHROCYTE MEMBRANES

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SUMMARY

Immunolatex spheres, originally developed as visual markers for scanning electron microscopy, were employed as membrane density perturbation reagents. Methacrylate spheres were bound to antibody molecules and used to label antigens on erythrocytes. Ghosts prepared from labeled cells were subjected to isopycnic centrifugation on continuous sucrose and dextran gradients. It was found that the labeled erythrocyte membranes had a substantially higher density than unlabeled membranes. The extent to which the membrane density was shifted on a given gradient depended on the number, size and density of the latex spheres and could be closely predicted by theory. These results suggest that the reagents and techniques described here have potential application for the isolation of plasma membranes from more complex cell types.

INTRODUCTION

One of the major difficulties encountered in purifying plasma membranes from animal cells has been in separating out other cellular membranes having similar physical properties. Differential and isopycnic centrifugation of cell homogenates have been used routinely to fractionate membrane fragments differing in size, shape and density [1]. Plasma membranes isolated by this methodology, however, have often been found to be contaminated with membranes derived from cellular organelles [2].

Recently, Wallach and coworkers [3] introduced a new membrane fractionation method, termed affinity density perturbation. In this technique, the density of membrane vesicles bearing lectin receptors was increased by the binding of concanavalin A-virus complexes.

In this paper we describe a modification of the affinity density perturbation

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method designed to specifically isolate plasma membranes. This technique utilizes immunolates, previously employed as visual markers for scanning electron microscopy [4, 5], to label cell surface antigens. The labeled cells are then disrupted and the homogenate is subjected to isopycnic centrifugation. Plasma membranes will band at a higher density on a continuous gradient due to the bound latex-antibody conjugates and hence can be separated from membranes with a lighter density.

We have used red blood cells as a model system to test the applicability of latex spheres to serve as membrane density perturbation reagents. The effect of the number, size and density of the latex spheres on the density of labeled erythrocyte membranes is reported.

MATERIALS AND METHODS

Methylmethacrylate, 2-hydroxyethylmethacrylate, methacrylic acid and ethyleneglycol dimethacrylate were obtained from Rohm and Haas and purified by vacuum distillation as previously described [5]. Glutaraldehyde (25 % aqueous solution) was purchased from Polysciences; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide from the Ott Chemical Co.; CNBr from Matheson, Coleman and Bell and dansyl- ϵ -lysine from Sigma Chem. Co. Rabbit IgG was obtained from Miles-Pentex and goat anti-rabbit γ -globulin antiserum from Antibodies Inc. Rabbit antiserum against human erythrocytes (rabbit anti-erythrocyte antiserum) having a hemagglutination titer of 2^{11} was obtained from Cappel Laboratories.

Phosphate-buffered saline, pH 7.40, was composed of 8.0 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4 and 1.15 g Na_2HPO_4 per l of distilled water. 310 mosM buffer according to Dodge et al. [6], pH 7.4, was prepared by dissolving 2.742 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 12.747 g of Na_2HPO_4 into 1 l of solution. The corresponding 20 mosM buffer was then prepared from the 310 mosM buffer by dilution.

Gradients were made with either ultra-pure sucrose (Schwarz-Mann), dextran of average molecular weight 40 000 (Sigma) or glycerol (Mallinckrodt).

Synthesis of latex spheres

Acrylic latex spheres consisting of 53 % methylmethacrylate, 30 % 2-hydroxyethylmethacrylate, 10 % methacrylic acid and 7 % ethyleneglycol dimethacrylate were synthesized by aqueous emulsion copolymerization as previously described [5]. Briefly, spheres having an average diameter of 100 nm (1000 Å) were prepared by adding 5.8 g methylmethacrylate, 3.3 g 2-hydroxyethylmethacrylate, 1.1 g methacrylic acid, 0.8 g ethyleneglycol dimethacrylate, 0.1 g of sodium dodecyl sulfate and 0.01 g of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ to 88.9 ml of water. This yielded a total methacrylate monomer concentration of 11 %. The reaction mixture was saturated with argon and polymerization was carried out at 98 °C for 1 h in sealed, tumbling containers. Copolymer spheres having a diameter of 30 nm (300 Å) were polymerized under similar conditions from a solution containing 3 % total monomer. Ionic impurities were removed by passing the latex suspension, adjusted to pH 7.0, through a mixed-bed ion-exchange column consisting of Biorad AG 1-X10 and AG 50-W-X12 resins.

Purification of goat anti-rabbit IgG antibodies

Goat anti-rabbit IgG antibodies were purified from whole serum on an immu-

noadsorbent column consisting of rabbit IgG coupled to Sepharose 4B by the CNBr method [7, 8]. NaSCN (3 M) in 0.01 M phosphate buffer, pH 7.0, was used to elute the goat anti-rabbit IgG from the column. The eluate containing the antibody was dialyzed exhaustively against phosphate-buffered saline over 3–4 days at 4 °C. Anti-rabbit IgG activity was assayed by immunodiffusion.

Preparation of fluorescent immunol latex spheres

Acrylic latex spheres were tagged with fluorochrome, dansyl- ϵ -lysine, using the CNBr reaction, and subsequently coupled to goat anti-rabbit IgG antibodies by a two-step glutaraldehyde reaction as previously described [5]. Typically, 10 ml of latex spheres (40–50 mg/ml) were activated with 100 mg of CNBr at pH 10.5 for 15 min at 25 °C. The suspension was then cooled to 4 °C and added to an equal volume of 10 mM dansyl- ϵ -lysine in 0.2 M sodium carbonate buffer at pH 10.0. The reaction was allowed to proceed overnight at 4 °C. Uncoupled dansyl- ϵ -lysine was removed by dialysis against several changes of 0.1 M NaCl over 4–5 days.

The fluorescent latex spheres were derivatized with diaminoheptane by adding 20 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide to 10 ml of latex spheres (20–30 mg/ml) in 0.01 M diaminoheptane at pH 7.0. The carbodiimide reaction was carried out at 4 °C for 2 h. Free diaminoheptane was removed by exhaustive dialysis against phosphate-buffered saline.

The diaminoheptane-derivatized spheres were activated with 1.25 % glutaraldehyde at 25 °C for 1 h. Excess glutaraldehyde was then removed by dialysis overnight at 4 °C against several changes of phosphate-buffered saline. 5 mg of goat anti-rabbit IgG antibodies were added to 10 ml of the glutaraldehyde-activated spheres and the mixture was stirred at 25 °C for 3–5 h. The reaction was stopped by the addition of 1 ml of 0.1 M glycine, pH 8.0.

Goat anti-rabbit IgG antibody-latex conjugates were separated from unbound antibody by centrifugation on a discontinuous sucrose gradient as previously described [5]. Conjugate solutions (20 mg/ml) were stored at 4 °C in tightly closed vials until used.

Preparation of erythrocyte ghosts and membrane vesicles

Type 0, Rh⁺ blood was collected into acid/citrate/dextrose solution. Erythrocytes were washed three times in phosphate-buffered saline by centrifugation (500 $\times$ g, 15 min). Erythrocyte ghosts were prepared by the procedure of Dodge et al. [6] and stored frozen at -20 °C in 0.1 M sucrose. Membrane vesicles were prepared from ghosts by sudden decompression in an Artisan pressure homogenizer [9] after equilibration with N₂ for 25 min at 700 lb/inch².

Labeling of cells and ghosts

Erythrocytes and ghosts were labeled by an indirect (sandwich) technique. Aliquots, containing 4–5 $\cdot$ 10⁸ erythrocytes, equilibrated to 4 °C, were pelleted (500 $\times$ g, 10 min) and resuspended into 25 μ l rabbit anti-human erythrocyte antiserum. In the control sample, 25 μ l of phosphate-buffered saline was substituted for the erythrocyte-specific antiserum. The cells were incubated for 1 h at 4 °C with occasional shaking to prevent settling and minimize formation of large aggregates. 3–4 ml cold phosphate-buffered saline were added and the cells were pelleted (400 $\times$ g, 20 min).

This washing procedure was repeated three more times. The final pellet was resuspended in 200 μl goat anti-rabbit IgG-latex conjugate (0.5–20 mg/ml), and incubated with occasional shaking for 30 min at 25 °C. Each sample was then diluted with 4–5 ml phosphate-buffered saline and centrifuged at $300\times g$ for 20 min. Supernatant containing unbound latex conjugates was removed. Cell samples were washed once more with phosphate-buffered saline and resuspended in 0.4 ml 310 mosM buffer according to Dodge et al. [6]. 25- μl aliquots were removed for scanning electron microscope studies. The remaining cells in each sample were lysed by the addition of 6 ml of 20 mosM buffer according to Dodge et al. [6]. The ghosts were pelleted at $6000\times g$ for 20 min, washed with 10 ml of the 20 mosM buffer, resuspended in 0.3 ml of phosphate-buffered saline and layered on the gradient for isopycnic centrifugation.

Frozen and thawed membranes were labeled similarly except that incubation in antisera was at 25 °C for 30 min and a higher g force ($20\,000\times g$, 10 min) was used to pellet the membranes. Unbound latex conjugates were not separated from labeled membranes prior to layering onto the gradients.

Determination of isopycnic density

Continuous linear density gradients of sucrose (25–58 %, $\rho = 1.11\text{--}1.26\text{ g}\cdot\text{cm}^{-3}$), dextran (5–40 %, $\rho = 1.02\text{--}1.17\text{ g}\cdot\text{cm}^{-3}$) or glycerol (20–80 %, $\rho = 1.05\text{--}1.20\text{ g}\cdot\text{cm}^{-3}$) were prepared with a Buchler density gradient mixer. All gradient solutions were prepared with phosphate-buffered saline. Samples were layered onto the gradients and centrifuged to equilibrium at 4 °C and $100\,000\times g$ in a Beckman SW 39L rotor. Typical centrifugation times were 1–1.5 h for sucrose gradients, 4 h for glycerol gradients and 14–15 h for dextran gradients or for small vesicles in sucrose. Prolonged centrifugation (16 h) in sucrose gradient resulted in aggregation of labeled membranes, but the final density was the same as that after 1 h of centrifugation.

Each gradient was fractionated into 150- μl fractions and the refractive index was measured to determine the density. The presence of membrane or latex conjugates was determined from light scattering measurements at 280 nm after dilution with 0.5 ml phosphate-buffered saline.

Determination of degree of labeling

Labeled erythrocytes were washed 3–4 times, fixed with 1.25 % glutaraldehyde in phosphate-buffered saline and centrifuged onto ionene-coated coverslips as previously described [5]. Samples were dehydrated in a series of ethanol solutions (50–100 %), critical-point dried from Freon 13, and coated with gold-palladium. Samples were examined with an ETEC scanning electron microscope operating at 20 kV.

The degree of labeling was determined from scanning electron micrographs by counting the number of latex spheres visible on one surface of labeled erythrocytes (including the rim). 4–8 cells from each sample were counted. The count for each sample was averaged and doubled and used as an estimate of the total number of latex spheres on the whole cell. This procedure tends to underestimate the total number since some latex spheres on the rim of the cell could be obscured.

RESULTS

Properties of erythrocyte membranes and latex-antibody conjugates

Erythrocyte ghosts prepared by lysis of human red blood cells in hypotonic solution [6] were biconcave in shape as viewed by phase contrast microscopy. Ghosts subjected to nitrogen cavitation [9] or sonication were disrupted into small vesicles having a wide distribution of sizes, generally less than $0.5\ \mu\text{m}$ in diameter. Protein composition of ghosts prepared by the procedure of Dodge et al. [6], as determined by gel electrophoresis, was identical to that reported by Fairbanks et al. [10].

The isopycnic densities of erythrocyte ghosts and vesicles in several gradients are summarized in Table I. The density of the ghosts depends on their permeability to the gradient material and on the effect of osmotic pressure [1, 11, 12]. Since membranes are permeable to glycerol [2], the observed density of ghosts in a glycerol gradient ($\rho = 1.187\ \text{g} \cdot \text{cm}^{-3}$) is essentially the hydrated density of the erythrocyte membrane. The densities in sucrose and in dextran gradients are lower than in glycerol and presumably correspond to the buoyant density of the ghosts subjected to the osmotic pressure induced by the non-permeating gradient material. This interpretation is in accord with the observations of Bodemann and Passow [13]. Their type II (sealed) ghosts and our ghosts have similar sucrose gradient densities.

TABLE I

ISOPYCNIC DENSITY OF ERYTHROCYTE GHOSTS AND LATEX CONJUGATES

Erythrocyte ghosts were prepared by the procedure of Dodge et al. [6]. Densities were then determined by equilibrium centrifugation at 4°C .

Sample	Gradient material	Density ($\text{g} \cdot \text{cm}^{-3}$)
Ghost	Glycerol	1.187
Ghost	Sucrose	1.169
Ghost	Dextran	1.088
Latex-antibody conjugates (100 nm diameter)	Sucrose	1.232
Latex-antibody conjugates (30 nm diameter)	Sucrose	1.232

The reduction in volume of the sealed ghosts (or vesicles) which arises from the osmotic effects of a non-permeant gradient can be calculated from the observed buoyant density (ρ_v) using the relation

$$\rho_v = \frac{\rho_m V_m + \rho_s V_s}{V_m + V_s} \quad (1)$$

where ρ_m is the hydrated membrane density (the isopycnic density in a permeating gradient, e.g. glycerol), V_m is the membrane volume, V_s is the volume of the fluid inside the vesicle at equilibrium and ρ_s is the density of the fluid inside the vesicle.

For erythrocyte ghosts in a sucrose gradient with $\rho_m = 1.187\ \text{g} \cdot \text{cm}^{-3}$, $\rho_v = 1.169\ \text{g} \cdot \text{cm}^{-3}$, $\rho_s = 1.0\ \text{g} \cdot \text{cm}^{-3}$, membrane surface area $A_m = 145\ \mu\text{m}^2$ [14],

membrane thickness $h_m = 7$ nm and $V_m = A_m \cdot h_m = 1.01 \cdot 10^{-12}$ cm³. V_s was calculated to be $1.08 \cdot 10^{-13}$ cm³. Since the mean corpuscular volume is $8.7 \cdot 10^{-11}$ cm³ [15], V_s is only 0.1 % of the original ghost volume.

A similar calculation in dextran ($\rho = 1.088$ g · cm⁻³) leads to a V_s of $1.14 \cdot 10^{-12}$ cm³, approx. 1.3 % of the original ghost volume.

The density of latex-antibody conjugates is also given in Table I. Differences in conjugate density with different size latex spheres were undetectable.

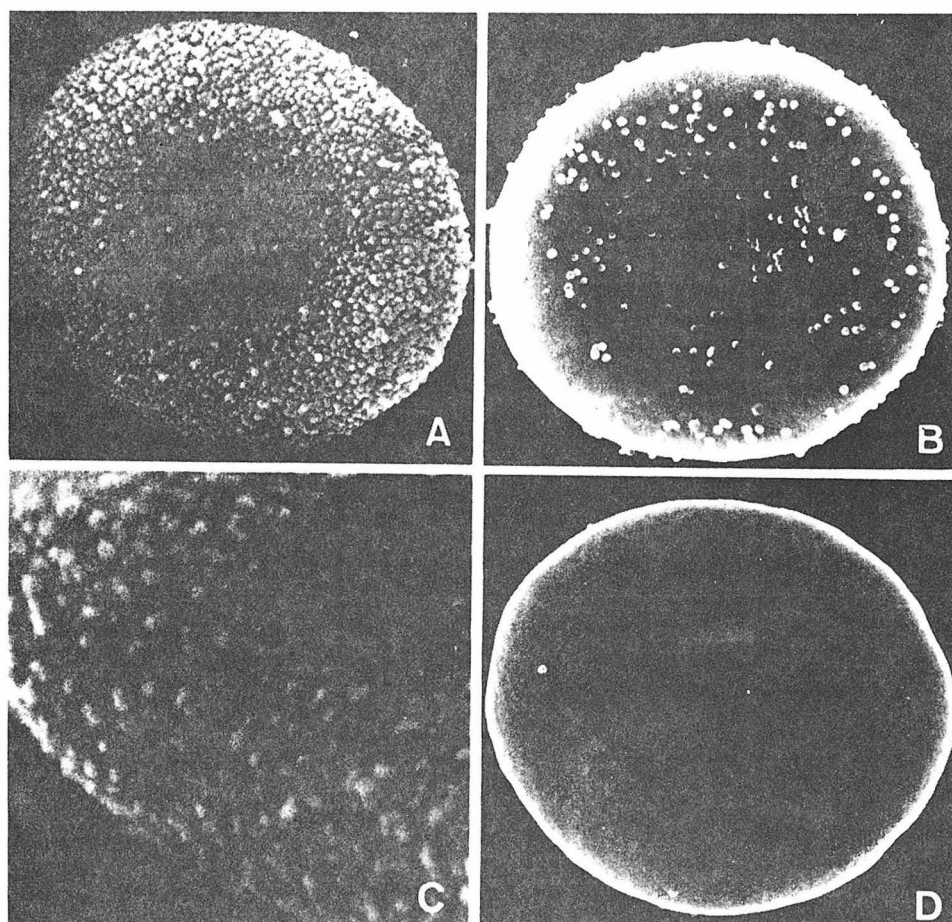


Fig. 1. Scanning electron micrographs of human erythrocytes treated with immunolatex conjugates. (A) Erythrocytes sensitized with rabbit anti-erythrocyte antibodies and subsequently labeled with goat anti-rabbit IgG antibody-latex conjugates at a concentration of 20 mg/ml. Spheres have an average diameter of 100 nm, but appear slightly larger in the micrographs due to the gold-palladium coating ($\times 9200$). (B) Erythrocyte treated as in A, but with a concentration of 0.5 mg/ml of immunolatex conjugate ($\times 9200$). (C) Erythrocyte at high magnification treated as in A, but labeled with immunolatex spheres (8 mg/ml) having a diameter of approx. 30 nm ($\times 50\,000$). These spheres, however, are more heterogeneous in size compared to 100-nm spheres (see ref. 5). (D) Control for A. Unsensitized erythrocyte incubated with 20 mg/ml of 100-nm diameter goat anti-rabbit IgG antibody-latex conjugate ($\times 9200$).

Erythrocytes labeled with immunolabel spheres

Erythrocytes sensitized with rabbit anti-erythrocyte antibodies can be labeled with goat anti-rabbit IgG antibody-latex conjugates for visualization by scanning electron microscopy [4]. Fig. 1a shows an erythrocyte densely labeled with immunolabel spheres having an average diameter of 100 nm (1000 Å). The number of spheres bound per cell depends on the concentration of the reagent used in the labeling procedure [4]: at low concentrations of immunolabel spheres, a sparse, random distribution of spheres is observed (Fig. 1b). Immunolabel spheres having a diameter of approx. 30 nm can also serve as markers (Fig. 1c). Non-specific binding of immunolabel spheres to unsensitized cells was extremely low (Fig. 1d).

When the labeled erythrocytes were hemolysed and washed by centrifugation, the dansyl-lysine tagged immunolabel spheres remained bound to the erythrocyte membranes as confirmed by fluorescent and scanning electron microscopy.

Density perturbation of erythrocyte membranes

As shown in the sucrose density gradients in Fig. 2, ghosts prepared from erythrocytes labeled with fluorescent immunolabel spheres band at a position between the unlabeled ghosts and the immunolabel conjugates. These membrane bands were intensely fluorescent under ultraviolet light, thus confirming the presence of bound

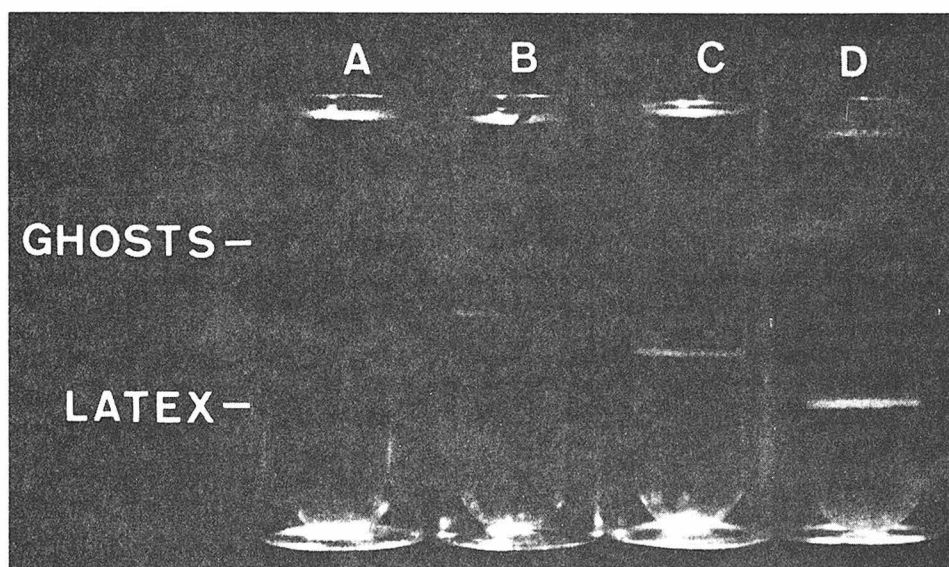


Fig. 2. The isopycnic banding of erythrocyte ghosts and latex spheres on linear sucrose gradients at 4 °C. Gradient A displays ghosts prepared from unsensitized erythrocytes treated with goat anti-rabbit IgG antibody-latex conjugate (100 nm in diameter). The measured density of $1.169 \text{ g} \cdot \text{cm}^{-3}$ is the same as that of erythrocyte ghosts not exposed to the conjugate. Gradient B shows ghosts derived from erythrocytes sensitized with rabbit anti-erythrocyte antibodies and labeled with immunolabel conjugate. A density of $1.204 \text{ g} \cdot \text{cm}^{-3}$ was determined and corresponded to 1795 spheres per cell. Gradient C shows ghosts derived from erythrocytes sensitized as in B, but treated with a higher concentration of conjugate. A density of $1.212 \text{ g} \cdot \text{cm}^{-3}$ was determined and corresponded to 3430 spheres/cell. Gradient D shows the sharp band obtained for latex spheres. A density of $1.232 \text{ g} \cdot \text{cm}^{-3}$ was measured.

latex reagents. The isopycnic density of the labeled ghosts depended on the concentration of immunolabel used to label the cells and, consequently, on the degree of labeling of the cells. The density of ghosts obtained from erythrocytes either labeled with rabbit anti-erythrocyte antibodies alone or else treated only with the immunolabel conjugates (control) was the same as that measured for ghosts of untreated cells. A similar dependence of density on conjugate concentration was observed when erythrocyte ghosts rather than intact cells were labeled.

The relationship between the number of spheres (100 nm diameter) bound per cell as counted in scanning electron micrographs (for example see Figs 1a and 1b) and the observed density of the labeled ghosts in sucrose gradients is depicted in Fig. 3. When 4500 spheres, having an average diameter of 100 nm, were bound per erythrocyte, a density of $1.215 \text{ g} \cdot \text{cm}^{-3}$ was achieved. When as few as 300 spheres were bound, a density change of 0.01 relative to unlabeled membrane (control) was found.

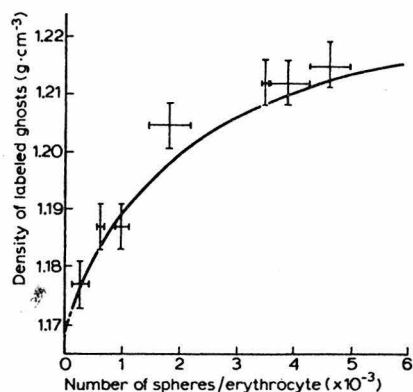


Fig. 3. The relationship between the number of immunolabel spheres (100 nm diameter) bound per erythrocyte and the isopycnic density of the corresponding labeled ghosts. Erythrocytes sensitized with rabbit anti-erythrocyte antibodies were labeled with varying concentrations of immunolabel spheres. The number of spheres was counted from scanning electron micrographs. The densities of the ghosts prepared from the labeled cells were determined by equilibrium centrifugation on linear sucrose gradients at 4°C . Data are given with standard deviations. Theoretical curve was calculated from Eqn 2.

Theoretically, the effect of the degree of labeling on the density of the latex particle-membrane vesicle complex (ρ_c) can be determined from the following equation:

$$\rho_c = \frac{\rho_m V_m + n\rho_b V_b + V_s \rho_s}{V_m + nV_b + V_s} \quad (2)$$

where n is the number of latex spheres bound to the vesicle and ρ_b , V_b are latex sphere density and volume, respectively. To a first approximation, V_s can be assumed to remain constant (i.e. identical to V_s of original unlabeled vesicles), then ρ_c can be plotted as a function of n . As shown in Fig. 3, there is a good correlation between the experimentally determined points and the calculated curve. Hence, any further

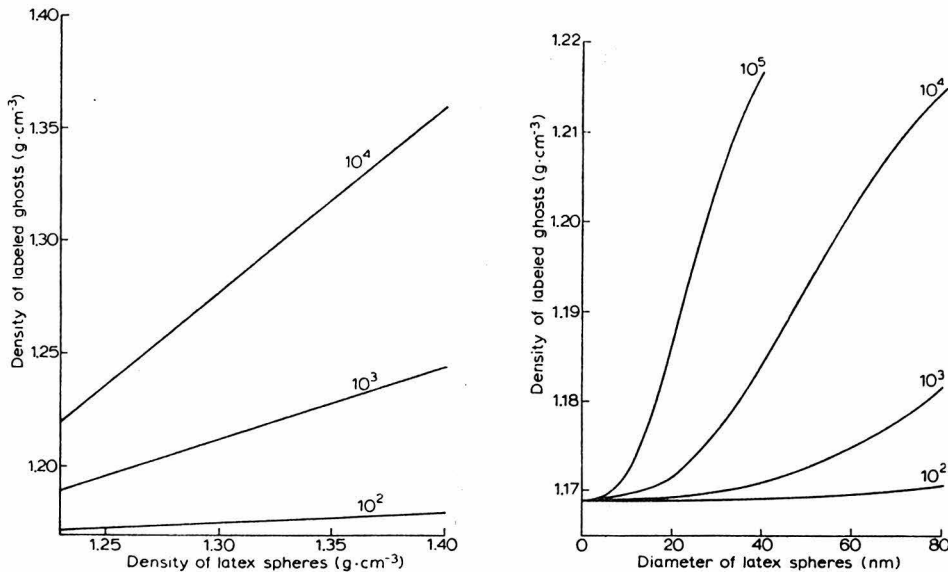


Fig. 4. Dependence of the density of labeled erythrocyte ghosts on the density and size of latex spheres. (A) Effect of increasing density of latex spheres on ghosts labeled with 10^2 – 10^4 spheres of 100 nm diameter. (B) Effect of different sizes of latex spheres on ghosts labeled with 10^2 – 10^5 spheres of density $1.232 \text{ g} \cdot \text{cm}^{-3}$. Curves were calculated from Eqn 2, using a membrane density = $1.187 \text{ g} \cdot \text{cm}^{-3}$, membrane thickness = 7 nm, and membrane area = $145 \mu\text{m}^2$.

decrease in V_s must be small. This is to be expected since unlabeled erythrocyte ghosts are already essentially collapsed at isopycnic equilibrium in sucrose.

Eqn 2 can also be used to predict the effect of changing various parameters on the isopycnic density of the membrane-latex complex. As shown in Fig. 4a, there is a linear increase in the isopycnic density of the complex (ρ_c) with an increase in the density of the latex spheres (ρ_b). The effect is especially pronounced with heavy labeling of the cell surface.

For a constant number of bound latex spheres, the isopycnic density of a labeled vesicle (ρ_c) will be higher for larger latex spheres (Fig. 4b). However, a significant density shift can still be obtained with small (approx. 30 nm diameter) spheres provided that heavy labeling occurs.

Table II summarizes the experimental results obtained when some of these parameters were varied. When 30-nm diameter spheres, instead of 100-nm spheres, were used to label cell surface antigens, a smaller density shift (difference between labeled and unlabeled ghosts) was observed. A greater density shift was found for labeled ghosts in dextran gradients. This difference is primarily due to the lower buoyant density of ghosts in dextran compared to that in sucrose gradients (Table I). The observed isopycnic density in dextran is in agreement with the value calculated from Eqn 2.

A preliminary study indicates that small membrane vesicles, both labeled and unlabeled, have a lower density than the corresponding intact erythrocyte ghosts. The membrane bands of small vesicles were also quite diffuse. The cause of this is

TABLE II

DEPENDENCE OF ERYTHROCYTE MEMBRANE DENSITY SHIFT ON VARIOUS PARAMETERS

Erythrocytes were labeled with immunolabel spheres of the indicated size and then lysed according to a modification of the procedure of Dodge et al. [6]. Vesicles were prepared from the erythrocyte ghosts with an Artisan pressure homogenizer. The densities of the membrane-latex complex were then determined as described in Table I. The number of spheres bound per cell was determined from scanning electron micrographs.

Experiment	Membrane sample	Gradient	Latex size diameter (nm)	No. cells counted	Average No. latex spheres/cell	Density shift ($\text{g} \cdot \text{cm}^{-3}$)
I	Ghost	Sucrose	100	5	3 476	0.041
	Ghost	Dextran	100	5	3 476	0.067
II	Ghost	Sucrose	100	4	3 322	0.035
	Vesicle	Sucrose	100	4	3 322	0.055*
III	Ghost	Sucrose	30	4	12 074**	0.023
Control	Ghost	—	100	6	10	—

* Vesicles showed up as a fairly diffuse band in the gradient. Density shift value corresponds to the density difference between the center of labeled and unlabeled vesicle bands ($\rho = 1.133 \text{ g} \cdot \text{cm}^{-3}$).

** The number of spheres in $2.5 \mu\text{m}^2$ of the cell surface was counted and the total number of spheres on the cell was estimated. This count is unreliable due to low resolution of the micrographs and the dense labeling.

uncertain, but may be related to an uneven distribution of latex spheres among the vesicles when ghosts were homogenized, osmotic effect on vesicles of different sizes, loss of protein or loss of some latex spheres from the membrane during the homogenization procedure. The last explanation is supported by the presence of a faint latex-conjugate band in the gradient containing labeled small vesicles but not in any other gradients. Nevertheless, as shown in Table II, the density shift is still quite significant and the homogenization procedure potentially can be improved to yield better results.

DISCUSSION

Methacrylate latex microspheres chemically bonded to antibody molecules have been used to label cell surface antigens by the indirect immunochemical method. In addition to serving as visual markers for scanning electron microscopy, these reagents increase the density of plasma membranes. The studies reported here using erythrocyte ghosts as a model system indicate that the extent to which the density of these membranes are shifted on a given gradient depends on the number, size and density of the latex spheres as predicted by theory.

These results suggest that plasma membranes from more complex cell types can be isolated using these latex spheres as density perturbation reagents. With a judicious choice of size and density of the spheres, as well as gradient material, labeled plasma membranes can be separated from other cellular membranes and hence, in principle, can be obtained in a highly purified state for biochemical charac-

terization. In cases where the number of cell surface binding sites for a particular latex-ligand conjugate is low, a substantial increase in membrane density can be achieved with relatively large (approx. 100 nm diameter) methacrylate spheres, as have been used in the present study. However, when a large number of sites are displayed on the cell surface, it is desirable to have small spheres (10–30 nm diameter) having a higher density ($\rho > 1.23 \text{ g} \cdot \text{cm}^{-3}$) for combined studies involving high resolution mapping of sites by electron microscopy and for isolation of plasma membranes by affinity density perturbation. Such spheres have been synthesized (Yen, S. P. S., unpublished) and will be described elsewhere.

Latex spheres chemically bonded to ligands which interact specifically and reversibly with cell surface components (e.g. lectins and hormones) are particularly well suited for the purification of plasma membranes by affinity density perturbation. Currently, we are investigating this approach and also exploring the applicability of these microspheres in the purification of specific cell surface receptors.

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REFERENCES

- 1 Steck, T. L. and Wallach, D. F. H. (1970) in *Methods in Cancer Research* (Busch, H., ed.), Vol. 5, pp. 93–153, Academic Press, New York
- 2 Wallach, D. F. H. and Lin, P. S. (1973) *Biochim. Biophys. Acta* 300, 211–254
- 3 Wallach, D. F. H., Kranz, B., Ferber, E. and Fischer, H. (1972) *FEBS Lett.* 21, 29–33
- 4 Molday, R. S., Dreyer, W. J., Rembaum, A. and Yen, S. P. S. (1974) *Nature* 249, 81–83
- 5 Molday, R. S., Dreyer, W. J., Rembaum, A. and Yen, S. P. S. (1975) *J. Cell. Biol.* 64, 75–88
- 6 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* 110, 119–130
- 7 Axén, R., Porath, J. and Ernback, S. (1967) *Nature* 214, 1302–1304
- 8 Cuatrecasas, P. (1970) *J. Biol. Chem.* 245, 3059–3065
- 9 Hunter, M. J. and Commerford, S. L. (1961) *Biochim. Biophys. Acta* 47, 580–586
- 10 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) *Biochemistry* 10, 2606–2617
- 11 de Duve, C., Berthet, J. and Beaufay, H. (1959) *Prog. Biophys.* 9, 325–369
- 12 Beaufay, H. and Berthet, J. (1963) *Biochem. Soc. Symp.* 23, 66–85
- 13 Bodemann, H. and Passow, H. (1972) *J. Membrane Biol.* 8, 1–26
- 14 Westerman, M. P., Pierce, L. E. and Jensen, W. N. (1961) *J. Lab. Clin. Med.* 57, 819–824
- 15 Wintrobe, M. M. (1967) in *Clinical Hematology*, p. 86, Lea and Febiger, Philadelphia

CHAPTER IV

Isoelectric Focussing of Rod Outer Segment Membrane Proteins

ISOELECTRIC FOCUSING OF ROD OUTER SEGMENT MEMBRANE PROTEINS

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1. Introduction

Isoelectric focusing has been employed extensively as a high resolution technique for the separation of water-soluble proteins [1]. The problems inherent in the solubilization of biological membranes, however, have limited the applicability of this method in the fractionation of membrane-bound proteins. In several such studies [2-4] membranes solubilized and focused in the presence of 6-8 M urea (and/or detergent) were resolved into a number of protein bands. Under such conditions which promote protein denaturation, however, identification of the proteins is difficult due to lack of suitable assay methods. Recently, using mild non-ionic detergents which preserve some of the 'functional' characteristics of membrane proteins the isoelectric focusing of *Rhodospseudomonas spheroides* Y reaction center [5] on polyacrylamide gels and the *Torpedo* acetylcholine receptor protein [6] on density columns have been described. Heitzmann and Richards [7] have mentioned the focusing of fluorescent derivatives of opsin.

In this report we describe a procedure for polyacrylamide gel isoelectric focusing of bovine rod outer segment membranes solubilized in the non-ionic detergent, Emulphogene (an alkoxypoly(ethylenoxy) ethanol). Under the conditions used the spectral characteristics and the light-dependent bleaching of rhodopsin are maintained. Furthermore, unbleached rhodopsin which focuses at pH 6.0 can be separated from opsin (bleached rhodopsin) which focuses at a lower pH. The finding that isoelectric focusing can separate different charged species of the same membrane protein points to the potential application of this method for the fractionation of proteins derived from other more complex membrane systems.

2. Materials and methods

Acrylamide, *N,N'* methylene-bis-acrylamide (Bis), *N,N,N',N'*-tetramethylethylenediamine (TMEDA) and ammonium persulfate (APS) were obtained from BioRad. The non-ionic detergent Emulphogene BC-720 was obtained from GAF. Ampholine, 40%, pH 3.5-10 (batch 1) was obtained from LKB-Produkter. All other chemicals were reagent grade.

2.1. Rod outer segments (ROS)

ROS were prepared from frozen bovine retinas (Hormel & Co., Austin, Minn.) under dim red light according to the procedure of McConnell et al. [8] with some modifications [9, 10]. The final ROS membrane suspensions at 6-8 mg/ml protein concentration and in 0.2 M sucrose were stored frozen at -20°C in small light-tight vials. Protein concentration was determined by amino acid analysis using a Beckman Model 121 Analyzer. Using a molar extinction coefficient at 500 nm of 4.05×10^4 and a mol. wt. of 3.9×10^4 [11], concentrations of unbleached rhodopsin were estimated from the absorbance measurements at 500 nm after solubilization of ROS in 2% Emulphogene and centrifugation at 20 000 g for 10 min. The purity of ROS preparations was verified by absorbance ratios: $A_{400} : A_{500}$ and $A_{280} : A_{500}$.

2.2. Sodium dodecyl sulphate (SDS) gel electrophoresis

SDS gel electrophoresis was performed by the method of Fairbanks et al. [12].

2.3. Isoelectric focusing

All solutions were freshly made using deionized distilled water. Manipulations involved in rhodopsin sample preparation and focusing were carried out under dim red light.

2.3.1. Gel preparation

For 12–13 gels, 1.5 g acrylamide, 0.05 g Bis, 0.075 ml Emulphogene were dissolved in 26.4 ml water. The solution was degassed for 5 min by stirring under vacuum. After the solution was chilled in an ice bath, 1.5 ml Ampholine, 1.0 ml 1.5% (w/v) APS and 1.0 ml 0.5% (v/v) TMEDA were added with stirring. The gel solution was quickly pipetted into acid-washed Pyrex gel tubes (125 × 8 mm i.d.), the lower ends of which were sealed with rubber grommets, and were overlaid with 0.05 ml of water. Gel tubes were filled to a height of 8 cm. Rapid polymerization necessitated fast working in pouring and overlaying the gel solutions to ensure flat gel tops. It was noticed also that Ampholine, without addition of TMEDA or APS, will initiate and propagate polymerization. The gels were stored overnight at room temperature before use.

This procedure yielded a polyacrylamide gel containing 5% acrylamide (with 1:30 Bis cross-linkage), 0.25% detergent and 2% Ampholine concentration. A slight shrinkage of the gels occurred during polymerization resulting in a final gel length of 7.8 cm. The gels had background A_{280} of 0.4 and were permeable to β -galactosidase (mol. wt. 530 000).

2.3.2. Sample preparation

ROS samples used for isoelectric focusing were diluted with an equal volume of water and pelleted at 20 000g (Sorvall RC-2B centrifuge, SS-34 rotor, 13 000 rpm) for 10 min. The pellet was solubilized in a carrier solution at pH 8 such that the final concentrations were 1% detergent, 2% Ampholine and 0.1–1.0 mg/ml protein. The solution was incubated at 4°C for 45 min and centrifuged at 25 000 g for 40 min to pellet any unsolubilized material. A few crystals of sucrose (Mann ultrapure) were dissolved in a known volume of the supernatant in order to increase the density of the solution and thus facilitate layering onto gels.

In some experiments the samples were bleached for varying lengths of time in a quartz cuvette at a distance of 7 cm from a 100 W light source equipped with an infrared filter. Fifty percent bleaching was obtained after 3–4 sec. The percentage of rhodopsin bleached was determined from absorbance difference at 500 nm.

2.3.3. Electrophoresis

Gels were placed in an electrophoresis apparatus with 0.01 M phosphoric acid (anolyte) in the lower reservoir and 0.02 M sodium hydroxide (catholyte) in the upper reservoir. A Buchler Model 3–1014A voltage and current regulated power supply was used. The gels were electrophoresed at 1 mA/gel for 1 hr to remove residual ammonium persulfate and to initiate formation of the pH gradient. Samples of vol. 0.01–0.1 ml were then applied to the tops of the gels. Focusing was performed at 12°C with $\frac{1}{2}$ mA constant current per gel until a voltage of 30 V/cm of gel was reached, at which point the voltage was kept constant at 30 V/cm of gel. The current was observed to drop to a small constant value (about 0.25–0.35 mA/gel). After 10 hr under these conditions and protein loadings, focusing was terminated and gels were removed and tattooed with a 25G needle dipped in India ink at 2 cm and 1 cm from top and bottom of gels, respectively.

2.3.4. Gel staining

Protein gels were stained by a solution containing 20% 5-sulfosalicylic acid, 25% isopropyl alcohol, and 0.025% Coomassie Blue R-250 (Schwarz Mann) for 35 hr and then destained in 20% acetic acid. Staining patterns were similar when gels were fixed in 20% 5-sulfosalicylic acid to elute out detergent and Ampholine and then stained by the method of Fairbanks et al. [12]. This latter method gave a higher background staining than the former. Both 10% trichloroacetic acid, when used as fixatives, gave excessively high background staining. Total staining and destaining time required was 45 hr. The pH of each band was estimated by normalization of all gel lengths to that of the pH gel.

2.3.5. pH gel and gel scanning

The pH gel (loaded with 25–50 μ l blank carrier solution) was tattooed at 3 mm intervals and hand-sliced into 3 mm pieces with a clean razor blade. Each piece was further sliced into quarters and soaked in 1 ml 0.1 M potassium chloride for 1 hr with frequent shaking. The pH of each piece was then determined to ± 0.01 pH unit using a Radiometer Model 25 pH meter with a scale expander.

Gels were initially scanned at 280 nm (protein absorption), 380 nm (bleached rhodopsin absorption),

and 500 nm (unbleached rhodopsin absorption), using a Gilford 2000 spectrophotometer with a gel scanning attachment, then bleached for 10 min at 16 cm from a GE 100 W frosted bulb, and finally scanned at 500 nm and 380 nm.

2.4. Proton change measurements

One vol of ROS (1 mg protein) was mixed with 1 vol of water and the suspension was centrifuged at 20 000 g for 10 min. The pellet was rewashed with an additional 2 vol of water. After solubilization in 1.2 ml of 2% Emulphogene and centrifugation at 20 000 g for 10 min, an aliquot of the solution was used to measure the rhodopsin concentration and a known volume was pipetted into a reaction vial at 4°C. The pH was adjusted to the desired value with 0.01 N NaOH solution. The ROS solution was bleached and the pH change was recorded. A quantitative estimate of the proton uptake or release as a result of bleaching was determined from the volume of standardized titrant required to back titrate the solution to its original pH value.

3. Results

3.1. Equilibrium conditions for isoelectric focusing

Under the conditions described proteins migrated to their final positions by 4–6 hr. Considerable band-sharpening occurred until 10 hr when equilibrium was considered to have been reached. The pH gradient, formed early during focusing, extended from about pH 4 to pH 8 with a reproducible linear range from pH 4.5 to pH 7.5. The final pH at which the protein complexes focused (their apparent isoelectric point) varied less than ± 0.2 units between different experiments and less than ± 0.1 units in duplicate gels with a given focusing experiment.

3.2. Purity of ROS membranes

Bovine rod outer segment preparations used in the present study had absorbance ratios $A_{280}:A_{500} = 2.3\text{--}2.6$ and $A_{400}:A_{500} = 0.24\text{--}0.26$ after solubilization in 2% Emulphogene or 2% cholate detergent. In one preparation, comparison of the rhodopsin concentration by spectral measurements with the total protein concentration determined by amino acid analysis indicated that greater than 85% of the protein in

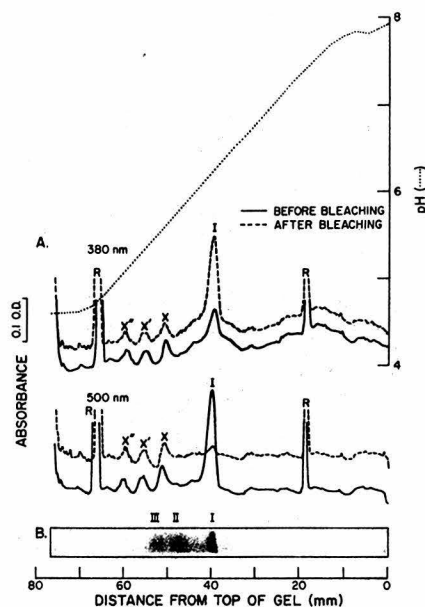


Fig. 1. Isoelectric focusing of unbleached ROS solubilized in 1% Emulphogene. A: pH gradient in the gel and spectrophotometric scanning traces of a gel before staining at 380 nm (bleached rhodopsin) and 500 nm (unbleached rhodopsin) before and after illumination. Small peak at 40 mm in 380 nm scan before bleaching is due to slight bleaching when gel was positioned for scanning. Bands II, III and minor bands are not detected by scanning. India ink reference marks (R) appear as sharp peaks at 20 mm and 70 mm. B: The same gel stained with Coomassie Blue. In this experiment bands I, II and III are at pH 6.2, 5.7 and 5.4, respectively.

ROS constitutes unbleached rhodopsin. Isoelectric focusing substantiated previous reports on the high content of rhodopsin in ROS membranes [11, 13, 14]. The electrophoretic pattern of ROS preparations on SDS-polyacrylamide gels is similar to that previously described [9] with rhodopsin exhibiting a relative mobility corresponding to an apparent mol. wt. 33–35 000.

3.3. Isoelectric focusing of ROS proteins

Rod outer segment membranes solubilized in the non-ionic detergent, Emulphogene, and focused in the dark on gels containing detergent displayed a band pattern shown in fig. 1. In the stained gels, one intense band (I) and two less intense bands, (II, III) were observed at pH 6.03 (S.D. 0.11), 5.62 (S.D. 0.13), and 5.26 (S.D. 0.14), respectively. Six faint bands were observed over the pH range 6.6–5.3. No bands were observed above pH 6.6.

The sharp major band I was identified as the visual receptor protein, rhodopsin, by spectrophotometric scanning of the gels before and after illumination prior to staining. This band was found to absorb light at 500 nm and to undergo bleaching upon exposure to light (fig. 1), resulting in the loss of absorption at 500 nm and an increase in absorption at 380 nm. The more diffuse band II did not absorb either at 500 nm or 380 nm.

The reproducible absorption-like peaks (X, X', X''), figs. 1 and 2) in the pH range 4.2–5.8 resulted from regions of constriction and swelling in the gel after focusing and also appeared in blank gels. It was found that these gel distortions were due to local low conductivities [15] presumably resulting from the properties of the particular Ampholine species present at these positions.

The effect of photobleaching on the isoelectric focusing pattern of ROS proteins is depicted in fig. 2. After focusing comparison of both the absorbance scans at 280 nm and the stained bands for unbleached, partially bleached (approx. 50%) and bleached ROS samples indicate that band I progressively decreases in intensity with increasing amount of bleaching, whereas the opposite is true for band II. On the basis of these results, we conclude that band II is opsin. The failure of this band to absorb 380 nm light is presumably due to the separation of the retinal group from opsin during electrophoresis. The isoelectric focusing patterns of a ROS sample which has been bleached 50% was the same as that for a sample containing an equal mixture of bleached and unbleached ROS.

No difference in protein band pattern was observed between ROS freshly prepared and ROS membranes stored at -20°C in the dark for over 9 months. In one ROS preparation, however, for which A_{280}/A_{500} was greater than 5, and additional intensely stained band was noticed at pH 5.0.

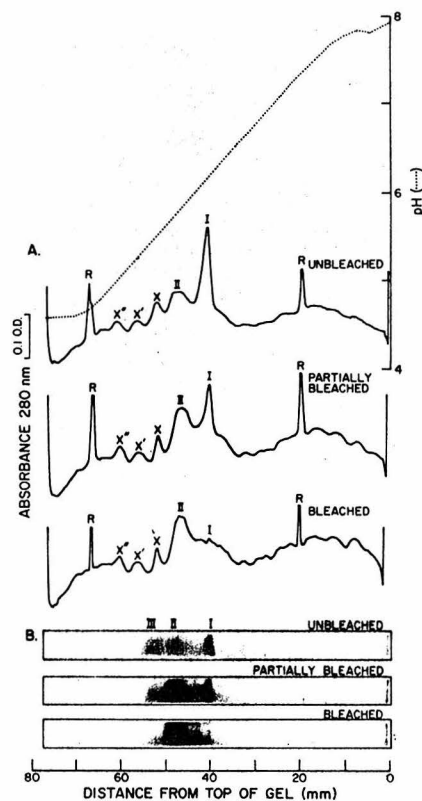


Fig. 2. Isoelectric focusing of unbleached, partially bleached (approx. 50%), and bleached ROS solubilized in 1% Emulphogene. A: pH gradient in the gels and spectrophotometric scanning traces of gels at 280 nm (proteins) before staining. Band III and minor bands are not detected by scanning. India ink reference marks (R) appear as sharp peaks at 20 mm and 70 mm. B: The same gels stained with Coomassie Blue. In this experiment bands I, II and III are at pH 6.2, 5.7 and 5.4 respectively.

3.4. SDS-gel electrophoresis

Bands I and II, eluted from the focusing gels and electrophoresed on SDS-gels, showed similar relative

mobilities to that of rhodopsin. These results support the conclusion that the major species of unbleached rhodopsin has an apparent isoelectric point of pI 6.0 and that after illumination it is lowered to pH 5.6.

From the loss in intensity of the stained band III on bleaching (fig. 2), it appears that this species is light-dependent, but its nature and significance are as yet unclear. The concentration of this species was too low to be measured by absorbance. The SDS-gel electrophoresis of this band when eluted showed a very faint band similar to rhodopsin in relative mobility. In all cases of SDS-gel electrophoresis Ampholine ran with the dye front and was stained.

3.5. Proton change upon bleaching.

At pH 8 a decrease of 0.35 pH units was observed upon photobleaching. This corresponded to a release of 2.5 protons per molecule of rhodopsin bleached. At pH 6 there was a 0.05 unit increase of pH. These data agree well with those of McConnell et al. [16] for ROS solubilized in Triton X-100.

4. Discussion

ROS preparations having demonstrated purity by spectral criteria have been focused on polyacrylamide gels. The results indicate that rhodopsin, the major component of ROS membranes, focuses at pH 6.0 in the presence of Emulphogene and can be resolved from opsin which focuses at pH 5.6 and other minor membrane proteins which band in the range pH 5-6.6. In this system the 500 nm absorption and light-dependent bleaching of rhodopsin are preserved.

The difference in apparent isoelectric points between unbleached rhodopsin and opsin is consistent with the presence of a greater net negative charge on the latter above pH 5.6. Two possible explanations for this effect are: 1) a release of protons due to a protein conformational change during bleaching in the detergent, and 2) a difference in the number of acidic phospholipids or other anions bound to the two forms of the protein. Quantitative measurements of proton changes during bleaching of detergent-solubilized ROS show a release of protons from rhodopsin bleached at pH 8 but a slight uptake of protons at pH 6. From this data and from the data of McConnell et

al. [16], the observed proton change at pH 6 does not account for the observed difference in isoelectric points on polyacrylamide gels. The apparent discrepancy between these two measurements may be a result of a difference in the nature of the protein complex before and after focusing. After solubilization of rhodopsin by nonionic detergents, different amounts of lipids can be further removed depending on the extraction procedure [17]. During focusing it is apparent that all-trans retinal is dissociated from opsin; it is also possible that lipids may be separated from rhodopsin and/or opsin, thus affecting their conformational states and the net change in protons upon bleaching. Related to this is the observation by McConnell et al. That upon bleaching ROS membranes exhibit an uptake of protons above pH 6, whereas Triton X-100-solubilized ROS show a release of protons. Heitzmann and Richards [7] reported that *N*-retinyl opsin, delipidated by a Brij 35-Triton X-100 mixture and subjected to isoelectric focusing, had an apparent isoelectric point of pH 5.4, similar to our results for opsin (pH 5.6). The more diffuse nature of the opsin band also suggests a heterogeneity in net charge, possibly a result of partial denaturation. Further studies on the effect of lipids on proton changes observed during bleaching and analysis of the lipid content of the rhodopsin and opsin protein bands from isoelectric focusing gels may yield further insight on these points.

The procedure of isoelectric focusing described here suggests the potential use of this technique for resolving phosphorylated forms of rhodopsin and thus for gaining information on the number and nature of the phosphorylated sites [9]. Furthermore, this technique may be useful in the fractionation and characterization of proteins of other membrane systems under mild conditions through appropriate choice of non-ionic detergents.

Acknowledgements

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References

- [1] LKB-Produkter AB, Acta Ampholinæ Literature Reference List (Solna Skriv & Stenografjänst AB, Solna).
- [2] Loach, P.A., Hadsell, R.M., Sekura, D.L. and Stemer, A. (1970) *Biochemistry* 9, 3127.
- [3] Merz, D.C., Good, R.A. and Litman, G.W. (1972) *Biochem. Biophys. Res. Commun.* 49, 84.
- [4] Fiechter, A., Mian, F.A., Ris, H. and Halvorson, H.O. (1972) *J. Bact.* 109, 855.
- [5] Jolchine, G. and Reiss-Husson, F. (1972) *Biochem. Biophys. Res. Commun.* 48, 333.
- [6] Eldefrawi, M.E. and Eldefrawi, A.R. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 1776.
- [7] Heitzmann, H. and Richards, F.M. (1973) *Biophys. Soc. Ann. Meet. Abstr.* 13, 232a.
- [8] McConnell, D.G. (1965) *J. Cell Biol.* 27, 459.
- [9] Kühn, H., Cook, J.H. and Dreyer, W.J. (1973) *Biochemistry* 12, 2495.
- [10] Papermaster, D.S. and Dreyer, W.J., manuscript in preparation.
- [11] Duemen, F.J.M., DeGrip, W.J. and Jansen, P.A.A. (1972) *Biochim. Biophys. Acta* 271, 419.
- [12] Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) *Biochemistry* 10, 2606.
- [13] Dreyer, W.J., Papermaster, D.S. and Kühn, H. (1972) *Ann. N.Y. Acad. Sci.* 194, 61.
- [14] Heitzmann, H. (1972) *Nature New Biol.* 235, 114.
- [15] Frater, R. (1970) *Analyt. Biochem.* 38, 536.
- [16] McConnell, D.G., Rafferty, C.N. and Dilley, R.A. (1968) *J. Biol. Chem.* 243, 5820.
- [17] Zorn, M. and Futterman, S. (1971) *J. Biol. Chem.* 246, 881.

APPENDIX

THE AREA-CODE HYPOTHESIS: THE IMMUNE SYSTEM PROVIDES CLUES
TO UNDERSTANDING THE GENETIC AND MOLECULAR BASIS OF CELL
RECOGNITION DURING DEVELOPMENT

By

L. Hood, H. V. Huang, and W. J. Dreyer

SUMMARY

Numerous studies of embryogenesis have provided evidence for highly specific cell-surface recognition phenomena. These include both the interactions of neighboring cells and the specific cellular migrations which occur as the developmental program of the embryo progresses. The area-code hypothesis elaborated here is an attempt to provide a framework for understanding cell-recognition phenomena in development.

This hypothesis is based on extensive genetic, molecular and cellular studies of the immune system. These studies suggest that the following events occur during the differentiation of antibody-producing cells.

- 1) Somatic cell lines of antibody-producing cells undergo a modification of their DNA as they become committed to synthesize a particular type of antibody molecule. This chromosomal modification event is probably a DNA translocation which leads to a somatic rearrangement of certain antibody genes.
- 2) In each of the specific cell lineages the new arrangement of DNA is inherited by all subsequent generations of cells.
- 3) The developmental programs which control these genetic alterations may be employed in a programmed and reproducible fashion. This programming of antibody development is suggested because different embryos appear to become committed to the production of identical antibody molecules in the same developmental sequence.
- 4) Antibody molecules are initially displayed on the cell surface where they serve as highly specific receptors to trigger the cell to proliferate and differentiate upon interacting with appropriate external molecular signals.
- 5) Antibody-producing cells display combinations of different molecules on their surfaces which cause each of

a very large number of different cells to interact differently with their environment. 6) The genes which code for many of these cell-surface molecules are organized into multigene families.

These observations as well as information from other developmental systems have led us to propose the area-code hypothesis. This hypothesis is concerned with the structure, function and regulation of cell-surface molecules that mediate recognition phenomena during embryogenesis. Area-code molecules are cell-surface molecules which are involved in the specific recognition phenomena during growth and development. These molecules provide cells with distinct cell-surface addresses or phenotypes, and provide the basis for the specificity in cell-cell recognition during cell migrations and cell-cell interactions, as well as serving as receptors for diffusible differentiation signals. The area-code hypothesis has three main postulates. i) There is a progressive display of specific combinations of area-code molecules on the surfaces of cells during development. ii) The genetic programs which determine the specific expression of area-code molecules are in part controlled by DNA modifications. These chromosomal modifications are believed to channel cells into specific lineages with progressively restricted developmental options. iii) Many of the area-code systems are organized into multigene families. Rapid evolutionary increases in complexity may proceed by the duplication and subsequent independent evolution of multigene families. In short, many of the remarkable events which occur during the development of the immune system may form a basis for understanding other developmental systems. Some experimental approaches toward testing this hypothesis are discussed.

Key words

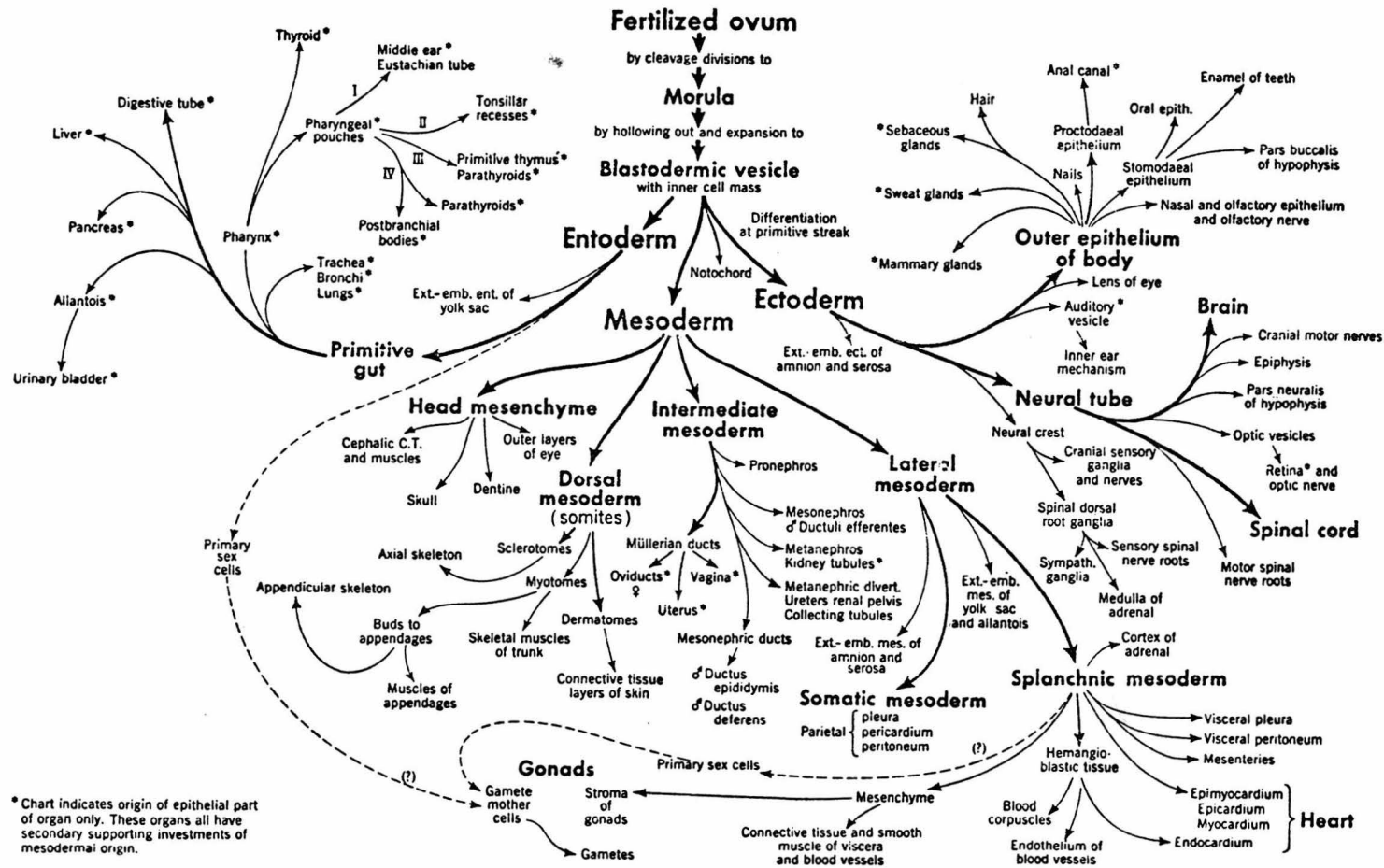
area-code hypothesis, combinations of cell-surface recognition molecules, chromosomal modifications, DNA translocation, multigene families, immune system as developmental model

INTRODUCTION

Development is the orderly process whereby a single cell, the zygote, generates a large diversity of cell types (Fig. 1). These cells migrate to appropriate locations and interact with one another to give rise to the supracellular organization of the adult organism. The adult human has about 10^{14} cells. If all cells were to divide at an equal rate, the average adult cell would be separated from the zygote by at least 48 cell divisions ($2^{47} = 10^{14}$). All adult cells have a cell lineage whose origin can be traced back to the zygote. As embryogenesis proceeds, cell lineages develop which become increasingly limited in their future development options (Fig. 1). Individual cells acquire specific developmental programs which limit the developmental fate of their progeny cells. Individual cells may become committed to a particular developmental program long before they differentiate to acquire the phenotypic characteristics of that cell lineage. These committed cells may later be induced to differentiate by hormones or other external signals. Little is known about the nature of the developmental programs or the mechanisms of cellular commitment. However, information has accumulated on the nature of cell-surface changes which occur as a cell lineage differentiates.

The unfolding of the developmental programs of individual cells leads to the expression of new gene products including molecules on the cell surface. Some of these cell-surface molecules are involved in cell recognition processes and may encompass a variety of functions in the developing embryo. Combinations of these molecules displayed on the surface may play a vital role in providing an address system for the

Figure 1. Cell lineages of a developing organism. From Patten, B. M., and Carlson, B. M. "Foundations of Embryology" 3rd ed., San Francisco: McGraw-Hill, 141 (1974). With permission.



massive cellular migrations that are characteristic of the developing embryo (Fig. 2). They are also vital to the myriad of specific cell-cell interactions occurring during growth and development. In the next section we propose a hypothesis that describes the general features of area-code molecules and the genes which encode them.

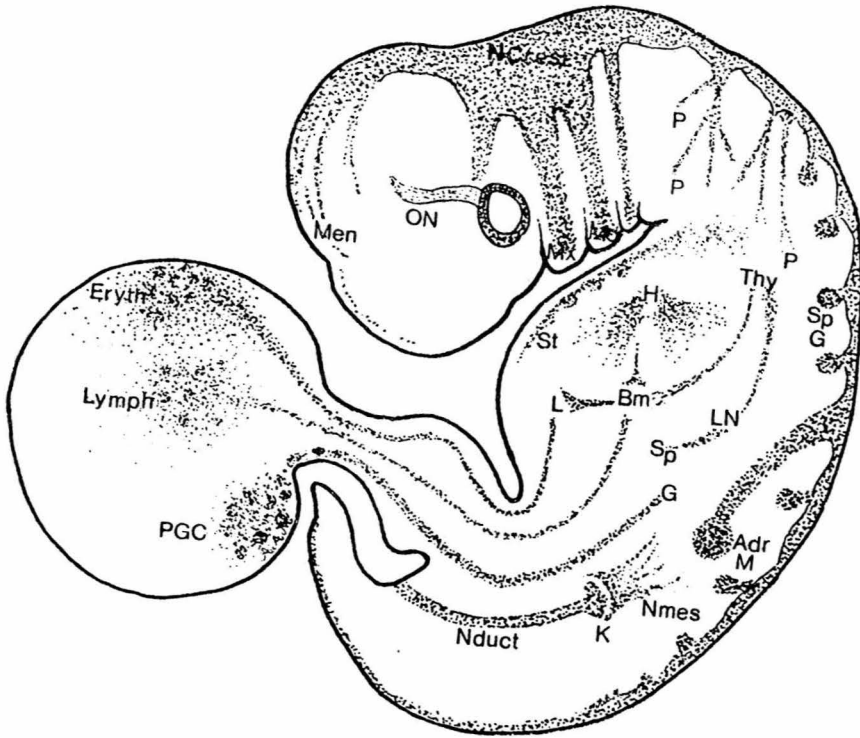
THE AREA-CODE HYPOTHESIS

The area-code hypothesis was formulated from an analysis of the vertebrate immune system. This hypothesis deals both with the role played by cell-surface recognition or area-code molecules in development and with the genetic events which place this address system on cell surfaces. The area-code hypothesis has several interrelated postulates: (i) During development combinations of area-code molecules are displayed on the surface of cells of specific lineages. These cell-surface displays provide the specificity for cell-cell interactions. They also provide cell-surface receptors essential for sensing diffusible differentiation signals such as those provided by hormones. Thus differentiating cells acquire distinct combinations of area-code molecules which serve as specific cellular addresses, not unlike those of the telephone or postal systems. (ii) The genetic programs which govern the expression of area-code molecules are controlled in part by alterations in somatic cell chromosomes such as the translocation of DNA sequences. These chromosomal modifications occur at branch points of differentiation which define cell lineages and limit the future options open to particular cell lineages. (iii) In many cases the genes encoding area-code systems are organized into

Figure 2. Cellular migrations in the embryo. This diagram displays events taking place at different stages of development.

	<u>Origin</u>	<u>Route</u>	<u>Target(s)</u>	
Erythrocyte precursors (Eryth)	Yolk sac	Blood stream	Liver (L), Bone marrow (Bm)	
Lymphoid cell precursors (Lymph)	Yolk sac	Blood stream	Bone marrow, Thymus (Thy), Lymph Nodes (LN), Spleen (Sp1)	
Primordial germ cells (PGC)	Yolk sac		Gonadal ridges, forming Gonads (G)	138
Mesenchyme cells	Dorsal embryo	Migrate ventrally	Form Sternum (St)	
Retinal ganglion cell axons	Eye	Optic Nerve (ON)	Brain visual centers	
Neural Crest cells (NCrest)	Neural crest	Migrate ventrally	Form Meninges (Men), embryonic skull cartilages (Mx, Md), Pigment cells (P), Spinal Ganglia (SpG),	

Heart cells	"Heart- forming territory"	Aggregate to	Form Heart (H)
Nephric ducts	Elongate and meet aggregations of Nephric mesenchyme cells (Nmes) to form Kidney rudiments (K)		
Not included in figure:	The intricate cell translocations in histogenesis of the nervous system, and other examples of cell translocations and aggregations (e.g., those resulting in the formation of hair follicles, teeth, limb rudiments).		



closely linked multigene families. During evolution, multigene families can be duplicated to produce new multigene families. These new families can assume new functions in programming development. Presumably these reprogrammed multigene families can produce major evolutionary changes in complex systems such as the brain. Thus the area-code gene families from diverse developmental systems may share common evolutionary origins.

It is our belief that area-code molecules play an important role in the recognition phenomena of embryogenesis for a variety of simple and complex systems including the neuroectoderm (1), the nervous system (2), the optic nerve (3), and the immune system.

THE IMMUNE SYSTEM IS A MODEL FOR STUDYING DEVELOPMENT IN COMPLEX EUKARYOTIC SYSTEMS

A. General Comments

The immune system is the first complex eukaryotic system that has been investigated in depth at the genetic, molecular and cellular levels. Its study has revealed some remarkable mechanisms and strategies for development that are embodied in the area-code hypothesis. Although some of these mechanisms appear unique to the immune system, we feel that this is so only because no other complex eukaryotic system has been studied in similar detail. We suggest that many of the developmental strategies of the immune system will be employed by other area-code systems.

The immune system has several features which facilitate its experimental study (4). These are summarized in Table I. The major cells of the immune system, lymphocytes, are freely wandering cells that can

Table I. Features of the Immune System Facilitating Its Study

1. Lymphocytes are freely wandering cells easily separated from other cell types and fractionated into functional subclasses.
2. Developmental decisions made in the immune system generate specific cell lineages in which the future options are genetically programmed.
3. Lymphocyte tumors represent clones derived from single progenitor cells. Many different stages of lymphocyte differentiation are represented in the available tumor lines.
4. Serologic reagents are available to identify different lymphocyte lineages. Thus specific tags are available for studying the differentiation, migration, interactions and triggering of lymphocytes.
5. The cells of the immune system undergo programmed migrations during development and interact in highly specific ways within various tissues such as the liver, spleen, bone marrow, thymus and lymph nodes.
6. Lymphocytes form a regulatory network with one another and with other cells. This network can be studied in vitro as well as in vivo. Thus it is perhaps easier to study cell cooperation in this system than in any other.
7. Triggering of lymphocytes to differentiate and divide by mitogens, antigens and hormones can readily be stud-

ied in vitro and in vivo.

8. Antibody molecules and mRNAs have been isolated from myeloma tumors and studied by chemical, serological, genetical, and functional techniques.
-

be readily separated from other cell types and even fractionated into functional subclasses. Migration pathways of embryonic lymphocytes can be traced as development proceeds in vivo. The differentiation of lymphocytes also can be followed in vitro. The availability of large quantities of antibody molecules and antibody mRNA from myeloma tumors has permitted a detailed analysis of the antibody molecules and their genes. The antibody molecule appears to play an important role in mediating cellular interactions among lymphocytes and thus serves as a model for the prototype area-code molecule. Cell-cell interactions and receptor-mediated triggering of lymphocytes to differentiate can be studied in vivo or in vitro. We will discuss in some detail the developmental, cellular, molecular, genetical and evolutionary strategies of the immune system as they have formed the basis for our thinking about the area-code hypothesis.

B. The Immune System Employs Antibody Molecules to Recognize Foreign Molecular Patterns

The vertebrate immune system shows two cardinal features of area-code systems, namely it utilizes recognition molecules exhibiting specificity and diversity.

Specificity. The immune system recognizes and destroys foreign molecules or antigens (5). The fundamental unit of recognition in this process is the antibody molecule. This molecule can be affixed to a lymphocyte as a specific cell-surface receptor or it can be secreted into the blood or lymphatic circulations. The antibody binds antigen through a molecular complementarity similar to that an enzyme exhibits

for its specific substrate. This interaction leads by a variety of mechanisms to the specific destruction or elimination of the antigen.

Diversity. Lymphocytes and their antibody molecules recognize a virtually limitless number of different antigenic determinants because almost any macromolecule that is foreign to a particular vertebrate organism can evoke an immune response. The average man has approximately 10^{12} lymphocytes circulating throughout his body and 10^{20} antibody molecules in his circulation. Estimates as to the number of different molecular species of antibody molecules a vertebrate can synthesize range between 10^5 and 10^8 . Hence the immune system is capable of generating an enormous array of different types of specific cell-surface recognition molecules. Let us consider how distinct cell lineages develop in the immune system.

C. Lymphocytes Differentiate Along One of Two Discrete Developmental Pathways to Produce B Cells and T Cells

Differentiation in lymphocytes requires specific cellular migration and hormonal induction, two features shared by other developmental systems.

B- and T-cell lineages. The development of the immune system begins with stem cells arising in the yolk sac and later migrating to the fetal liver and finally to the bone marrow (Fig. 2) (6). In the adult, stem cells for lymphocytes divide in the bone marrow and there become committed to the B- or T-cell pathway. These are termed pre-B or pre-T cells. Later these committed lymphocytes migrate to an

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appropriate microenvironment where hormonal inducers trigger the subsequent expression of the precommitted B- or T-cell developmental programs.

The pre-T cells migrate to the thymus. Under the influence of thymic hormones, they differentiate and later enter the circulation as mature T cells (7). T cells undergo additional differentiation steps on interaction of antigen with the antibody-like receptors on their cell surface.

In birds, the pre-B cell migrates to the bursa of Fabricius and presumably under hormonal induction differentiates to a mature B cell (8). In mammals, the pre-B cell probably differentiates in the bone marrow (9). B cells migrate to the blood and lymphatic circulation. There the interaction of antigen with antibody receptors induces terminal differentiation to the plasma cell, a highly efficient factory for the synthesis of antibody molecules.

B and T cells both employ antibody or antibody-like molecules as specific cell-surface receptors (10). The library of T-cell antibody-like receptors is believed to be comparable in diversity to those of its B-cell counterpart.

Functions of B and T cells. B cells synthesize antibody molecules. These molecules are employed as cell-surface receptors and they are also secreted into the serum. B cells constitute the basis of the humoral immune response which depends on the secreted antibody molecules to fight acute viral and bacterial infections. T cells synthesize antibody-like molecules which are employed as cell surface receptors for the diverse reactions of the cellular immune response. These include the surveillance for and destruction of cells altered by neoplastic transformation or

viral infection. T and B cells display characteristic cell-surface molecules, some of which mediate cell-cell interactions.

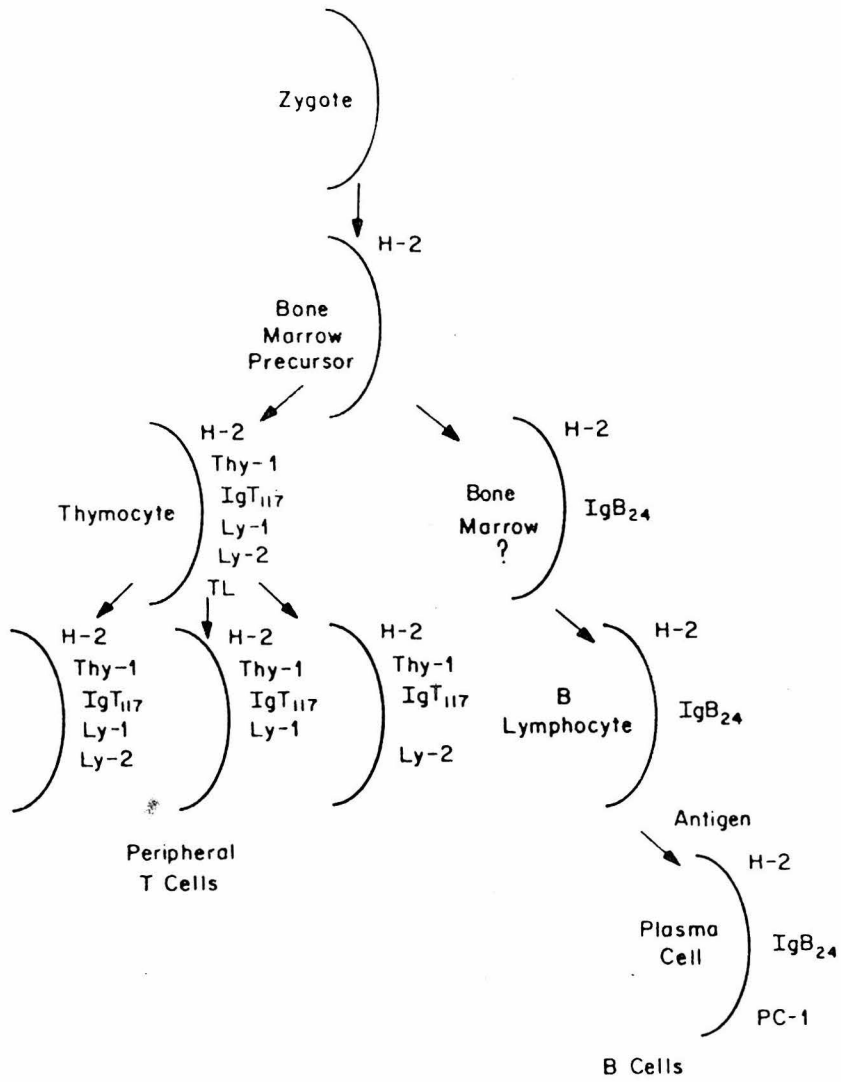
D. As Lymphocytes Differentiate, Distinct Combinations of Cell-Surface Molecules are Displayed

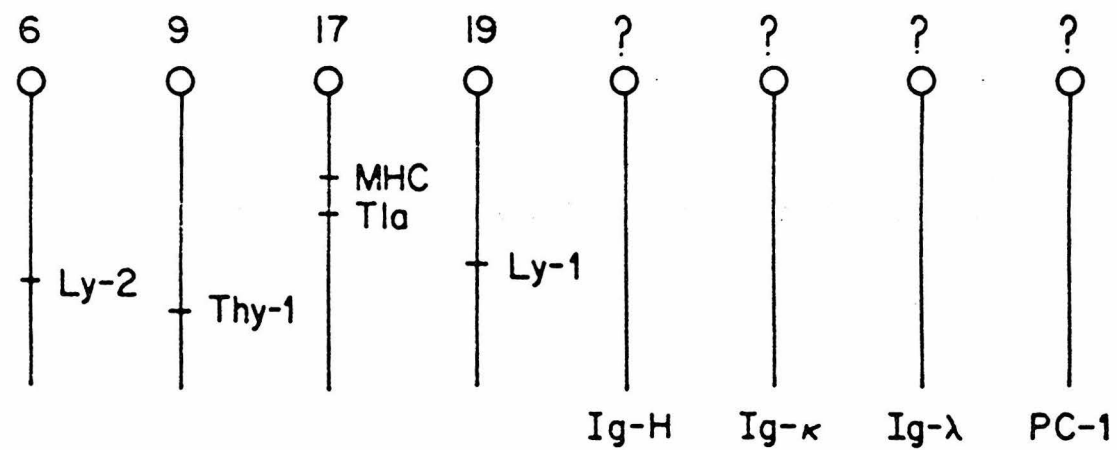
The area-code hypothesis suggests that cell-surface recognition molecules play a fundamental role in growth and development. The successive acquisition of cell-surface molecules during differentiation has been clearly demonstrated in lymphocytes.

Cell-surface molecules on T and B cells. The display of a variety of cell-surface molecules on mouse lymphocytes at various stages of development has been studied by detailed genetical and serological analyses (11). In the bone marrow all precursor cells of lymphocytes express the transplantation (H-2) antigens (Fig. 3). The pre-T cell migrates to the thymus and there is induced by thymic hormones to express at least four cell surface molecules: TL, Ly 1, Ly 2, and Thy 1 (12). The T-cell receptors, designated IgT, probably appear at this stage. As the T cell migrates to the periphery, TL disappears, the cell-surface concentration of Thy 1 decreases while that of H-2 increases (13). There are three Ly phenotypes of T cells in the periphery circulation, Ly 1, Ly 2, and Ly 1,2, and it appears likely that the Ly 1 and Ly 2 cells are derived from the Ly 1,2 cells (4). The pre-B cell migrates to the bursa or its equivalent and there presumably acquires the antibody receptor, designated IgB (Fig. 3). Antigen induces the expression of the PC-1 antigen in plasma cells (14).

Figure 3. Differentiation antigens on mouse lymphocytes.

The bone marrow lymphocyte precursor, which gives rise to the T- and B-lymphocyte lineages, expresses H-2 antigens on its surface. In the respective inductive microenvironments, lymphocytes display new sets of differentiation antigens. On seeding to the periphery some differentiation antigens may be selectively lost (e.g., TL, Ly 1 or Ly 2). Terminal differentiation may be correlated with new differentiation antigens (e.g., PC-1). H-2 designates the H-2 K and D transplantation antigens. Thy-1 denotes Thymus-derived lymphocyte antigen. IgT and IgB designate the antigen receptors of T and B cells, respectively, with subscripts of arbitrary numbers used to emphasize the great diversity of these molecules, and to stress the one cell-one antigen-binding site feature of these cells. Ly 1 and Ly 2 represent lymphocyte antigens 1 and 2 which are present exclusively on T cells. TL denotes thymus leukemia antigen. PC-1 represents plasma cell antigen. Other known lymphocyte differentiation antigens (e.g., Ia, Qa-2, Ala-1) are not shown. b) Chromosomal map of genes coding for differentiation antigens on mouse lymphocytes. The positions on the chromosomes are approximate for loci whose position is known. Chromosomal assignment for the immunoglobulin (Ig) families (H, λ , κ) and PC-1 are not known.





These cell-surface molecules are designated differentiation antigens (11) because they are molecules that have been expressed during successive developmental stages and have been studied by serological techniques.

Expression of differentiation antigens. Differentiation antigens show various modes of expression. i) The differentiation antigens induced in pre-T cells by thymic hormones can be expressed within five hours of induction. Moreover, this expression can occur without cell division (12). Accordingly, inducers trigger the expression of previously committed developmental programs. ii) Certain differentiation antigens are lost during the course of subsequent differentiation (e.g., TL), and others change markedly in their cell-surface concentrations (e.g., Thy 1 and H-2). Hence genes coding for differentiation antigens can be turned off or altered in their rate of expression.

* Sets of genes determine phenotype. The genes encoding the differentiation antigens of lymphocytes are present on at least eight different chromosomes (Fig. 3b). Thus, an array of cell-surface molecules encoded by unlinked genes determines the cell-surface state of differentiation in B and T cells. Different subsets of these genes must be expressed in a coordinated fashion by the developmental programs for each distinct cellular phenotype.

Differentiation antigens and lymphocyte-lineage relationships. Differentiation antigens provide clues as to the lineage relationships of various lymphocyte clones. T cells can be readily distinguished

from B cells based on their cell surface molecules (Fig. 3a). Indeed, three distinct subclasses of T cells can be distinguished in the peripheral circulation based on the Ly phenotypes (Fig. 3a). Finally, clones of lymphocytes acquire their unique functional specificities based on their expression of individual antibody or antibody-like cell-surface receptors. Thus, each individual lymphocyte clone expresses a unique set of cell-surface molecules beginning, for example, with those shared by all lymphocytes (e.g., H-2), to those shared by T cells (e.g., Thy 1), to those shared by T-cell subclasses (e.g., Ly 1, Ly 2 or Ly 1,2), and finally to those conferring clonal individuality (e.g., IgT_{117}) (Fig. 3). These combinations of cell-surface molecules serve as molecular addresses to distinguish individual clones of lymphocytes.

Area-code molecules and differentiation antigens. Area-code molecules are defined as those involved in cell-surface recognition processes. On the other hand, the differentiation antigens are any cell-surface molecule that appears during the course of differentiation in a particular cell lineage. Some differentiation antigens may be area-code molecules, though presumably not all. Many differentiation antigens may carry out cell-surface roles unrelated to cell recognition such as enzymatic reactions, structural support, and transport functions. The functions of the differentiation antigens of lymphocytes, apart from those of the IgT or IgB receptors, are unknown. Since the B-cell system serves as a model for an area-code system, let us consider how it is stimulated to undergo the final stages of differentiation by antigen.

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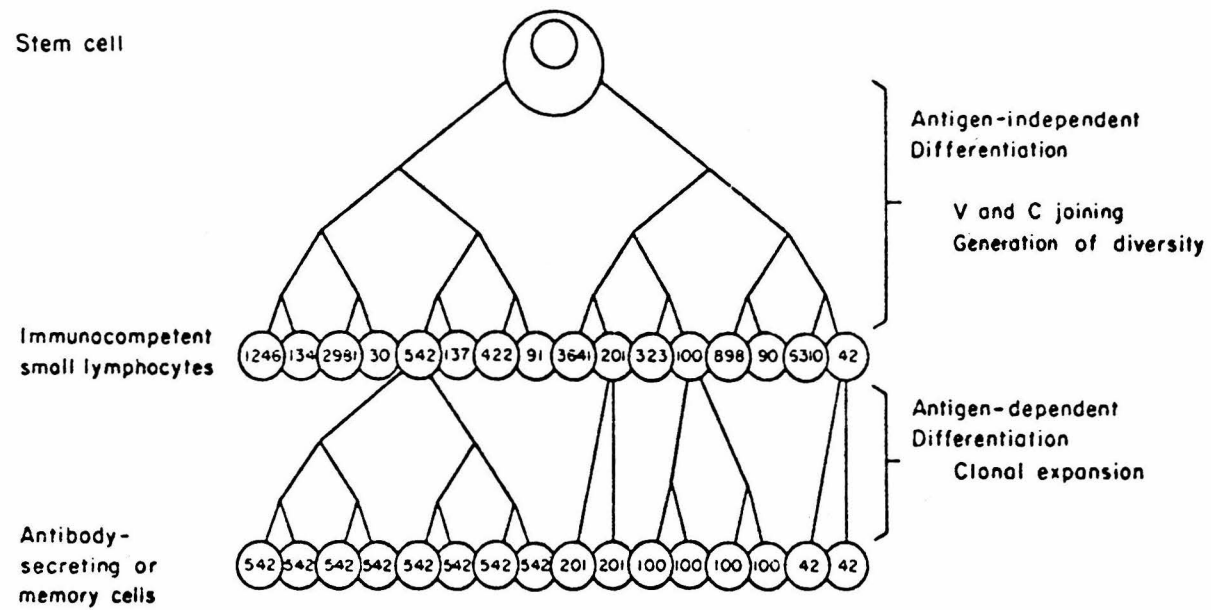
B. Antigen Triggers Clones of Lymphocytes with Complementary Antibody Receptors

One of the unresolved questions about complex eukaryotic systems employing cell-surface recognition molecules is how are they triggered to differentiate. While the molecular details of this triggering process are not understood for lymphocytes, a reasonable phenomenological description of this process is available.

In a system that encompasses 10^{12} lymphocytes expressing 10^5 to 10^8 different antibody molecules, how are appropriate antibody molecules expressed in response to individual antigens? There are several aspects to the triggering of a specific immune response to antigen (15) (Fig. 4). i) Individual lymphocytes can synthesize only one molecular species of antibody molecule. This commitment of each lymphocyte to the synthesis of one type of antibody molecule is an antigen-independent process. ii) Antigen triggers the clonal expansion of individual lymphocytes through interaction with complementary antibody receptors at the cell surface. iii) The clonal descendants of a particular lymphocyte are all committed to the expression of antibody molecules of precisely the same specificity as those of the parent lymphocyte. iv) Antigenic triggering of a lymphocyte results in two general classes of clonal descendants. Effector cells are terminally differentiated and mediate the immediate response to antigen. Memory cells constitute a greatly expanded specific lymphocyte compartment for enhanced secondary immune responses on re-encounter with antigen. Thus antigen is one of the final inductive triggers in lymphocyte differentiation. Moreover, antibody molecules, the prototype area-code molecules, play a fundamental role in this differentiation process.

Figure 4. A model for clonal selection in the immune system.

Adapted from (34).



Once antigen triggers clones of specific lymphocytes, antibody production from the individual lymphocyte clones must be regulated. Specific antibody molecules and antigen play an important role in this process. The effects of lymphocytes interacting with one another in a regulatory network are also very important in regulating the immune response.

F. Lymphocyte Interactions Regulate the Immune Response

One might argue that the immune system fails as a model for many potential area-code systems because lymphocytes are mobile and do not exhibit the fixed cellular interactions characteristic of most differentiated tissues. However, lymphocyte interactions with one another and with other tissues play a fundamental in lymphocyte differentiation.

Lymphocyte interactions in the immune response. Clones of lymphocytes interact with one another to regulate the immune response and produce a finely balanced lymphocyte network (16,17,18). The cellular bases for these interactions rests in part on the presence of three functionally distinct subclasses of T cells with differing Ly phenotypes (19). The Ly 1 helper-T cells cooperate with B cells or other T cells to produce an immune response. The Ly 2 cells fall into two distinct categories. The Ly 2 suppressor-T cells inhibit immune responses of B cells or other T cells. The Ly 2 killer-T cells destroy foreign cells by lysing them through unknown mechanisms. The Ly 1,2 cell also plays a role in the suppression process. These cellular interactions are summarized in Table II. Indeed, a third type of cell, the macrophage, also interacts with T cells to mediate certain aspects of the immune response (20). Macrophages

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Table II. Cellular Interactions Among Sets of Lymphocytes⁺

Cooperating Sets

Effector	Inducer	Function
B cell	Ly 1:H*	↑ antibody
Ly 2:KS*	Ly 1:H	↑ cytotoxicity
Ly 1,2:ARC*	Ly 2:KS	↑ suppressing
Macrophage	Ly 1:H	Delayed hypersensitivity
Macrophage	Ly 2:KS	Macrophage killers

⁺Adapted from (31).

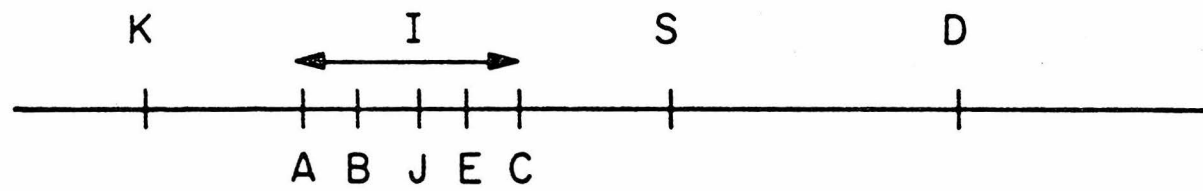
*H indicates helper; KS denotes killer-suppressor; and ARC designates antigen receptor cell.

share a common progenitor lineage with lymphocytes and, accordingly, are closely related in an embryological sense (21). Thus lymphocytes interact with one another and with macrophages to facilitate or suppress immune responses in a delicately balanced network of cellular regulation. Accordingly, lymphocyte networks may be an ideal model system for studying various aspects of cell-cell interaction -- a cardinal feature of development during embryogenesis and of the area-code hypothesis.

The lymphocyte network and cell-surface molecules. Lymphocyte interactions are mediated by several classes of cell-surface molecules including those coded by the major histocompatibility complex (22) and the antibody genes (17).

(1) In the mouse, the major histocompatibility complex, or H-2 complex, was defined on the basis of its ability to mediate strong graft rejections (13). This chromosomal complex encodes at least two different categories of cell-surface molecules (Fig. 5). First, the K and D regions encode the classical transplantation antigens, cell-surface glycoproteins which appear to play a fundamental role in T-cell surveillance of infected or transformed cells (23). Second, the I region encodes a number of cell-surface glycoproteins collectively designated the Ia antigens (see [24]). These molecules are present on B cells and, at lower concentrations, on at least some T cells. The I region regulates a bewildering array of immune-related traits. They include the control of the ability of mice to respond to a wide variety of different antigens (immune responsiveness) and some of the cell-surface interactions of T cell-B cell cooperation. The role of the Ia antigens in these functions is uncertain.

Figure 5. A genetic map of the major histocompatibility complex or H-2 complex of the mouse.



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Both the transplantation antigens and the I region gene products mediate specific lymphocyte interactions. The key to understanding these interactions is the realization that the genes of the K, D, and I regions are extremely polymorphic in mice. For example, inbred mice have at least eleven different K alleles and ten different D alleles (25). Thus cells with one combination of K, D, and I phenotype can be tested against those with other combinations. Such experiments demonstrate that in order for specific lymphocyte interactions to occur, these gene products must be identical on the two interacting cell types (23). For example, killer-T cells and target cells must share the same K or D gene products in order for the destruction of infected or transformed cells to occur. Also, helper-T cells must share I region identity with the cells that stimulate their participation in the immune response (26). The molecular basis for these cell-cell interactions is uncertain, however, it does not appear to be a simple like-like interaction of identical molecules coded by the H-2 complex. Thus, gene products from the K, D and I regions are involved in cellular recognition in the immune system and, accordingly, are area-code molecules.

(ii) The antibody molecules displayed on B cells and the antibody-like molecules on T cells also mediate cellular interactions among lymphocyte clones. The collection of antigenic determinants on the receptor portion (V domain) of an individual antibody molecule is known as its idiotypic (27). An idiotypic defines serologically each distinct molecular species of antibody molecule. Three interesting observations related to cellular interactions in the immune system have been made about idiotypes. First, animals can make antibodies to their own idiotypes (anti-idiotypic antibodies)

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(28). Second, anti-idiotypic antibodies may enhance or suppress immune responses (29). Third, the network of B cells, helper-T cells and suppressor-T cells involved in a particular immune response exhibit antibody receptors with related idiotypic and anti-idiotypic specificities (30). Thus cellular interactions in the immune response also are mediated by idiotypic-anti-idiotypic interactions. Since the antibody molecule participates in cellular recognition phenomena, it is also an area-code molecule.

(iii) Specific cellular interactions occur between lymphocytes and the stromal and endothelial cells of various lymphoid organs (31). For example, the lymph nodes and the spleen appear to have specific regions to which B cells or T cells migrate. Indeed, lymph nodes appear to have three separate regions, respectively, for B cells, Ly 1 and Ly 2 cells (32). Presumably specific sets of lymphocytes migrate to these areas because of specific cell-surface reactions with the underlying endothelial or stromal cells.

In summary, cellular interactions as well as precise cellular migrations are fundamental features of the immune system. Lymphocytes form a network of interacting cells that presumably play a fundamental role in regulating the immune response. Moreover, lymphocytes can specifically interact with other cell types such as macrophages and the stromal or endothelial cells of specific lymphocyte regions. Many of these interactions are mediated by cell-surface molecules. The programs for differentiation that lead to these specific cellular interactions are contained in T cells, in B cells and in the other cell types with which lymphocytes specifically interact. Clearly these programs and their expression of appropriate area-code molecules must be coordinated with one another to produce these functionally interacting cellular networks.

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Since the antibody molecule serves as a model for our thinking about area-code molecules, it is important to understand the molecular strategies it employs for its functions.

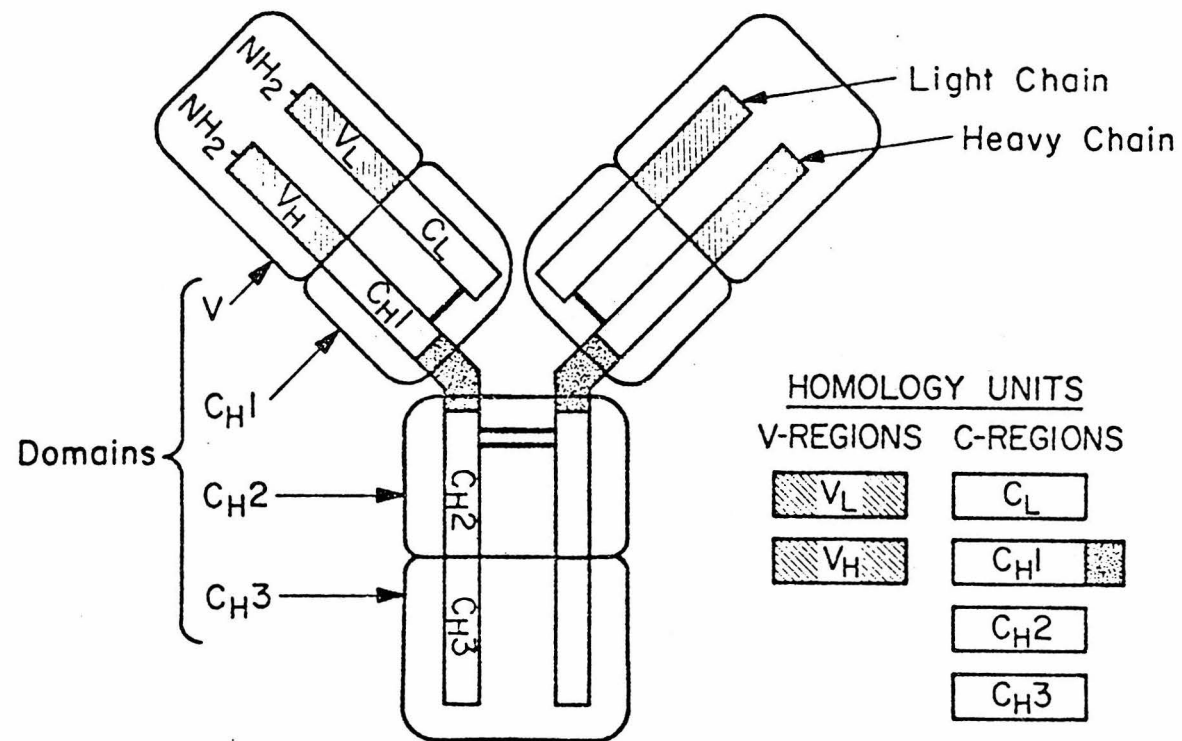
G. The Antibody Molecule is Composed of Discrete Globular Domains Which Carry Out Distinct Functions

Light and heavy chains (33). Individual antibody molecules are composed of two identical light and two identical heavy polypeptide chains associated by noncovalent and disulfide interactions (Fig. 6). Each antigen-binding site requires a single pair of light and heavy chains.

Variable and constant regions (34). All immunoglobulin polypeptides can be divided into an amino-terminal portion, the variable (V) region, and a carboxy-terminal portion, the constant (C) region (Fig. 6). The V regions exhibit extensive amino acid sequence diversity and the C regions far more limited diversity. The variable regions encode the antigen-binding or recognition function, whereas the C regions encode a more limited number of effector functions such as complement fixation.

Homology units (35). Antibody polypeptides can be divided into homology units about 110 residues in length on the basis of amino acid sequence similarities. The heavy chain in Fig. 6 is comprised of four homology units (V_H , C_H^1 , C_H^2 , C_H^3) and the light chain has two (V_L and C_L). The V_H and V_L homology units exhibit extensive sequence homology with one another as do the C region homology units. These homology relationships indicate that the homology units of antibody genes share a common evolutionary ancestry.

Figure 6. Structure of an antibody molecule. From (74).



Domains (35). Pairs of homology units (e.g., V_H-V_L , $C_H^1-C_L$, $C_H^2-C_H^2$ and $C_H^3-C_H^3$) fold into a compact globular domain (Fig. 6). The V_H and V_L homology units fold together to form a large crevice for antigen binding, whereas individual C domains carry out various effector functions. For example, the carboxy-terminal domain affixes the antibody molecule to the lymphocyte cell surface and is involved in the antigen-stimulated triggering of differentiation. Likewise, the C_H^2 domain mediates complement fixation (36). Thus the antibody molecule is a sophisticated molecular machine that folds into discrete globular domains each of which may carry out different functions.

The antibody molecule has fused together two discrete types of functions -- recognition and effector. This functional dichotomy of the antibody molecule is reflected in the structure and organization of the antibody genes.

H. Antibody Genes are Encoded as Three Multigene Families with Two Distinct Types of Genes -- Variable and Constant

The organization of the antibody gene families provides important insights into the possible organization of gene families for other area-code systems.

Three gene families. Classical genetic studies have demonstrated that three clusters or families of antibody genes, λ , κ and H, for B cells are present in all mammals studied to date (Fig. 7). These gene families are genetically unlinked to one another. The λ and κ gene families code for

Figure 7. Organization of the antibody gene families in man.

Adapted from (34).

Kappa Family $\downarrow \frac{V_{\kappa 1}}{\downarrow} \parallel \frac{V_{\kappa 2}}{\downarrow} \parallel \frac{V_{\kappa 3}}{\downarrow} \parallel \dots \parallel \frac{V_{\kappa m}}{\downarrow} \parallel \dots \parallel \frac{C_{\kappa}}{\downarrow} \parallel$

Lambda Family $\downarrow \frac{V_{\lambda 1}}{\downarrow} \parallel \frac{V_{\lambda 2}}{\downarrow} \parallel \frac{V_{\lambda 3}}{\downarrow} \parallel \frac{V_{\lambda 4}}{\downarrow} \parallel \dots \parallel \frac{V_{\lambda n}}{\downarrow} \parallel \dots \parallel \frac{C_{\lambda 1}}{\downarrow} \parallel \frac{C_{\lambda 2}}{\downarrow} \parallel \frac{C_{\lambda 3}}{\downarrow} \parallel \frac{C_{\lambda 4}}{\downarrow} \parallel$

Heavy Family $\downarrow \frac{V_{H 1}}{\downarrow} \parallel \frac{V_{H 2}}{\downarrow} \parallel \frac{V_{H 3}}{\downarrow} \parallel \dots \parallel \frac{V_{H p}}{\downarrow} \parallel \dots \parallel \frac{C_{\mu 1}}{\downarrow} \parallel \frac{C_{\mu 2}}{\downarrow} \parallel \frac{C_{\mu 4}}{\downarrow} \parallel \frac{C_{\gamma 2}}{\downarrow} \parallel \frac{C_{\gamma 1}}{\downarrow} \parallel \frac{C_{\gamma 3}}{\downarrow} \parallel \frac{C_{a 2}}{\downarrow} \parallel \frac{C_{a 1}}{\downarrow} \parallel \frac{C_{\theta}}{\downarrow} \parallel \frac{C_{\epsilon}}{\downarrow} \parallel$

light chains whereas the heavy gene family codes for heavy chains. Thus multiple gene families encode the antibody-receptor molecules.

Antibody gene families are multigenic (38). The number of antibody genes present in the germ line (or zygote) of a vertebrate organism is still a matter of controversy. However, most immunologists agree that the average antibody gene family has somewhere between 20 and thousands of genes. The antibody gene families are multigenic in nature.

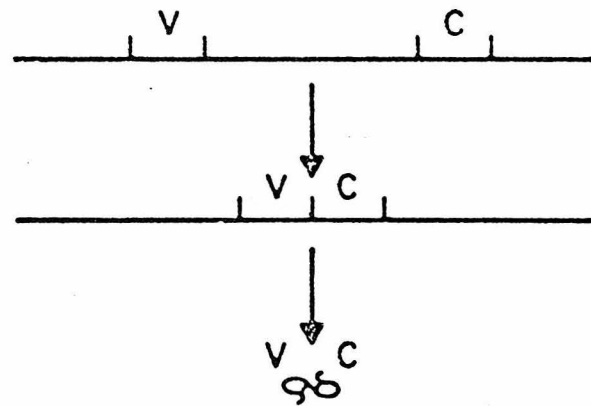
Separate V and C genes. The variable and constant regions of antibody polypeptides appear to be encoded by separate germ line genes (Fig. 7). The evidence for this surprising gene organization is compelling. Initially this supposition was based on genetical, serological, and amino acid sequence analyses (40). Subsequently this problem has been approached directly by the use of restriction endonucleases which cleave DNA at specific recognition sites (41,42). When genomic DNA is digested by such an enzyme and the resulting fragments are separated by size, a particular gene present as a single copy in the genome will be found only in one or a few fractions. Accordingly, linkage relationships between two or more genes (or the lack thereof) can be determined by examining these fractions with appropriate nucleic acid probes (radio-labeled mRNA or cDNA). In this manner, it has been shown that the V and C genes for light chains from the mouse are in separate restriction fragments in undifferentiated mouse embryo DNA, but on the same restriction fragment (and presumably joined) in differentiated myeloma tumor DNA. More recently, DNA sequence analysis of a V gene isolated from mouse embryonic DNA has confirmed that the C gene is not adjacent to the V gene in undifferentiated DNA (43). Obviously, the separate organization of V and C genes has important implications for mechanisms of lymphocyte differentiation.

I. The Translocation of V and C Genes in Lymphocytes during their Differentiation Appears to be a Fundamental Mechanism of Commitment

V-C translocation and antibody polypeptides. Since the V and C genes are separated in the embryo, these genes must undergo a rearrangement during somatic differentiation to form a contiguous VC gene that is translated into a single polypeptide chain. This probably occurs by a DNA translocation event that joins the V and C genes (Fig. 8).¹

V-C translocation and cellular commitment. Each mature lymphocyte expresses one type of antibody molecule. We feel that DNA translocation is a fundamental component of the molecular mechanism for committing a single lymphocyte to the expression of one type of antibody molecule (44) (Fig. 8). The implications of this hypothesis are extremely interesting with regard to development. The new arrangement of genetic material, including the V and C genes which have been joined as a part of the developmental process, becomes a heritable property of the daughter cell lines. The process of joining a specific V and a C gene is a definitive example of a commitment event. It provides a simple mechanism for limiting the future options open to a given cell lineage. It also provides a mechanism to explain how the memory of developmental decisions can be maintained throughout cell division and passed on to subsequent generations of somatic cell lines of the same lineage. The new arrangement of DNA in differentiated lymphocytes is simply replicated and passed to the daughter cells along with the newly activated genetic programs which control future events in the lineage.

Figure 8. Illustration of the differentiation of a lymphocyte through V-C joining. From (38).



genes - undifferentiated

genes - differentiated

polypeptide

V-C translocation may alter developmental programs. The V-C translocation during somatic differentiation in lymphocytes has two distinct functions. First, it joins together separate V and C genes into a single, contiguous V-C sequence which codes for a complete antibody chain. Second, it changes the developmental program of the lymphocyte so that it is hereafter committed to the expression of a single antibody polypeptide. Thus V-C translocation may alter the organization of control as well as structural elements and thereby alter the future developmental options of that particular cell lineage. We view this DNA modification event as a fundamental feature of differentiation in the antibody system and as we shall discuss subsequently in the differentiation of other area-code systems. Moreover, DNA translocation may be involved in the orderly read out of antibody genes during development.

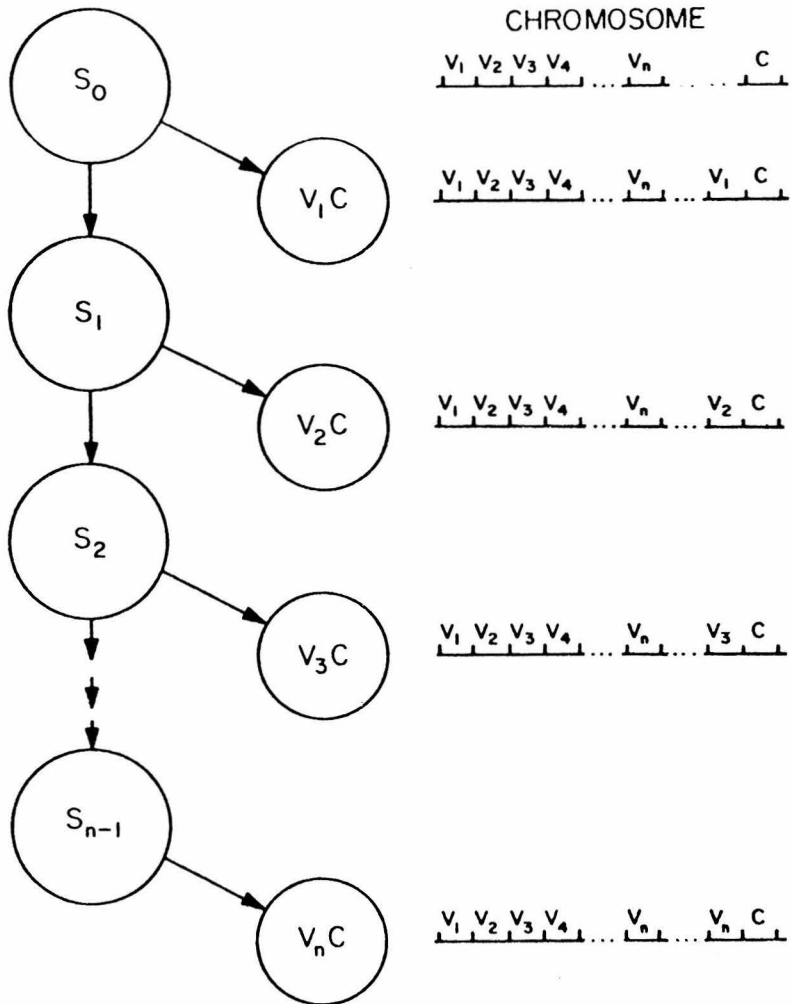
J. Antibody Genes may be Expressed during Differentiation in an Orderly and Programmed Manner

Development requires the orderly expression of phenotypic information. For example, during the differentiation of the T-cell lineage, the differentiation antigens are expressed at precise developmental stages (Fig. 3a). Accordingly, the set of unlinked genes that codes for these molecules (Fig. 3b) must be expressed in a coordinated and orderly manner.

Antibody V genes may also be expressed during differentiation in a coordinated and orderly manner (Fig. 9). Although the issue is controversial (see 45), several lines of evidence suggest that animals acquire the ability to respond to different antigens at different stages of development of the immune system. i) The development of individual clones of specific

Figure 9. A model of the linear and programmed read out of V genes in a multigene family.

STEM CELL LYMPHOCYTE



antibody-producing cells can be followed using X-irradiated mice as an in vivo tissue culture system (46). When lymphocytes are taken from neonatal mice, the ability to respond to the dinitrophenyl and trinitrophenyl groups appears by the first day after birth, to fluorescein by three days after birth, and to phosphorylcholine at six to seven days after birth (47). All mice in an inbred line seem to follow this same developmental progression. Moreover, isoelectric-focusing analyses of these antibody molecules suggest that the same major molecular species are expressed in each mouse at each developmental stage. ii) In the bursa of Fabricius of individual chicken embryos, specific antigen-binding lymphocytes for keyhole limpet hemocyanin and poly-L(Tyr,Glu)-poly-D,L-Ala-poly-L-Lys appeared earlier than those binding sheep erythrocytes (48). Once again this implies that certain antibody molecules are expressed during differentiation before others. (iii) Since the bursa appears to be central organ for B-cell differentiation in chickens, an attempt has been made to remove this organ at various stages of chicken development and determine whether there is a corresponding loss of the ability to express certain antibody polypeptides (Huang and Dreyer, in preparation). Early ablation should delete most of the antibody repertoire, whereas later ablations should permit the reproducible expression of an ever increasing fraction of the total repertoire. Preliminary studies of this type employing the very sensitive technique of two-dimension gel electrophoresis bear out these predictions and further suggest that there may be an ordered and reproducible expression of light chains.

Each of these individual studies can be given alternative interpretations (45), but taken together they raise the intriguing possibility that antibody genes may be read out during development in a programmed and orderly

fashion (Fig. 9). If so, several interesting questions are raised. During the differentiation of lymphocytes, are the V genes in a given multigene family sequentially translocated to their corresponding C genes? Is the V-C translocation mechanism an integral part of the development program for reading out V genes? Is there some type of mechanism for coordinating the read out of two multigene families (e.g., light and heavy chain gene families) so that particular molecular combinations of these two distinct polypeptide chains may be expressed in a programmed and reproducible fashion? How might genetic translocation help set up future developmental programs? Clearly those area-code systems mediating cell-cell recognition during embryogenesis must be capable of expressing their information in an orderly and programmed manner consistent with the orderly nature of growth and development.

K. Antibody Genes Appear to Evolve from a Common Precursor Gene

The evolution of antibody genes gives important insights into the possible evolutionary pathways of other area-code systems and raises the intriguing possibility that some distinct area-code systems may share common gene ancestors.

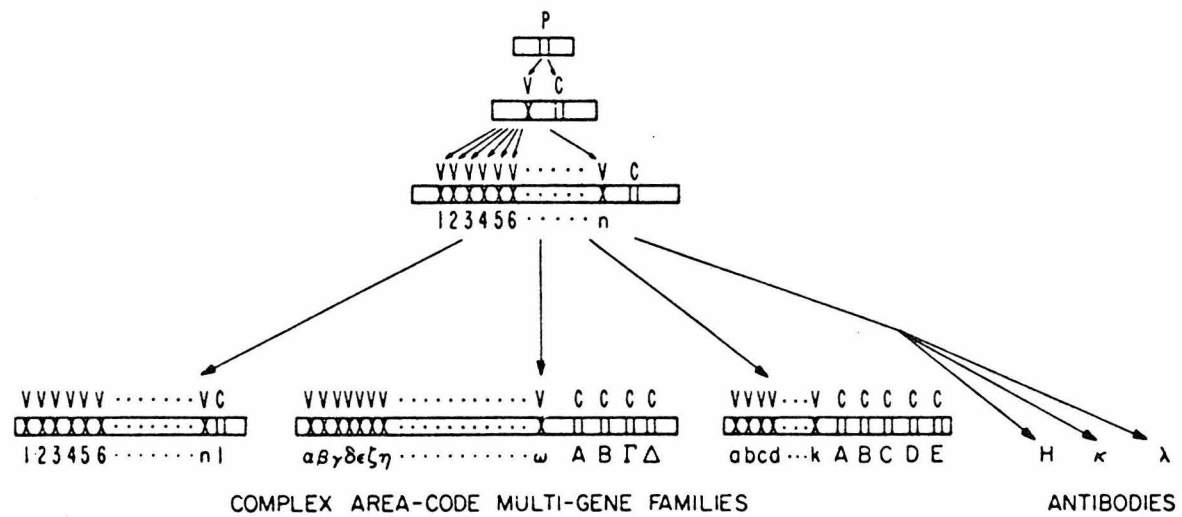
Antibody polypeptides are linearly differentiated into homology units of about 110 amino acid residues (Fig. 6). The constant homology units demonstrate significant amino acid sequence homology to one another as do the variable homology units (35). The existence of variable

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and constant region homology units and the observation by X-ray crystallographic analysis that the tertiary structures for the V and C homology units are very similar (49,50) indicate that antibody genes probably evolved from a precursor gene coding for a single ancestral homology unit. One hypothetical evolutionary scheme is depicted in Figure 10. The hypothetical precursor gene duplicated at a very early time to produce ancestral V and C genes. These gene products presumably assumed primitive area- code functions on membranes. Once a V-C translocation mechanism evolved, the V gene library could be expanded to generate a primordial multigene family.

This original family may have coded for primitive membrane area- code molecules (Fig. 10). This multigene family was in turn duplicated either by polyploidization or duplication and translocation of a chromosomal fragment to produce primitive antibody gene families. Subsequent duplication of this primitive multigene family produced the three families that evolved to become contemporary λ , κ and H families. Contiguous or fused gene duplication in the heavy chain family led to the different C_H genes, each comprised of three or four homology units. Homology units, folding to comprise domains with different functions, can be added to (or deleted from) C_H genes in the course of evolution. Accordingly, the evolution of antibody genes employed all the major mechanisms of gene evolution -- point mutation, discrete duplication, contiguous duplication, polyploidization and/or translocation. As we shall discuss subsequently, the antibody gene families may share a common evolutionary origin with other cell-surface recognition systems (Fig. 10).

Figure 10. A hypothetical model for the evolution of the antibody and other area-code gene families.



L. The Immune System Employs a Variety of Mechanisms for the Amplification of Information

An enormous amount of information is required to develop a eukaryotic organism. Thus it is of interest to analyze those strategies the immune system employs for the amplification of information. Once again, many of these strategies will almost certainly be employed by other area-code systems.

The vertebrate immune system can respond specifically to a universe of different antigenic determinants by virtue of molecular interactions with complementary antibody molecules. How then can the gene products from a finite number of antibody genes react with untold numbers of different antigenic determinants? The basic strategies for the amplification of antibody information fall into two broad categories -- genetic and molecular (Table III). Genetic strategies amplify information by producing multiple antibody genes, whereas molecular strategies amplify information by employing certain fundamental characteristics of antibody molecules themselves. i) The antibody gene families are multigenic and thus encode directly multiple receptor molecules (38). ii) Somatic mutation may occur in germ line antibody genes during the differentiation of individual lymphocytes to produce additional antibody genes (52,53). iii) At the genetic level different V genes may associate with the same C gene (e.g., V_1C_μ , V_2C_μ ... V_nC_μ). In addition, the same V gene may associate with different C genes (e.g., V_1C_μ , $V_1C_{\gamma 1}$, $V_1C_{\gamma 2}$, $V_1C_{\gamma 3}$... V_1C_ϵ). Thus the combinatorial-joining mechanism of DNA translocation allows one library of recognition sites to be combinatorially associated with a second library of effector functions. Moreover, during

Table III. Levels at which Information Amplification Occurs

Genetic Level

1. Multiple germ line genes
2. Somatic mutation
3. Combinatorial joining of V_H and C_H genes
4. Association of different homology units by crossing over during evolution.

Protein Level

1. Combinatorial association of subunits
2. Multispecificity

Cell-Surface Displays

1. Combinations of two or more distinct species of cell-surface molecules
-

evolution crossing-over can add (or delete) homology units to heavy chains. Thus various combinations of domains can be associated in a single molecule by evolutionary mechanisms. iv) The diversity of antigen-binding sites can be increased by the combinatorial association of light and heavy chains. For example, if 10^3 different L chains could associate with 10^3 different heavy chains, 10^6 different antibody molecules will be produced ($10^3 \times 10^3 = 10^6$). Thus unrestricted light and heavy chain interactions will generate an amplification factor of $p \times q$, where p equals the number of light chains and q the number of heavy chains. v) Multispecificity is defined as the ability of a single antibody molecule to interact with a variety of different antigens, some presumably related in tertiary structure and others possibly unrelated. Thus the inherent degeneracy of the antigen-binding site is an important mechanism for amplifying the information contained in a discrete number of antibody V genes. vi) At the supramolecular level, cell-cell recognition phenomena may involve combinations of two or more distinct species of cell-surface molecules. For example, T-cell surveillance of virus transformed cells requires the simultaneous recognition of both viral and transplantation antigens on the transformed cells (23). One explanation for this simultaneous dual recognition of two distinct molecules is that the viral antigen and the transplantation antigen associate at the cell surface to form a supramolecular complex. Obviously, the combinatorial association of cell-surface molecules can lead to significant amplification of the number of distinct cell-surface recognition units.

Thus the immune system displays a variety of strategies at the genetic, protein, and surface-display levels for the amplification of information (Table III). Other membrane recognition systems will certainly employ similar strategies.

M. Summary of Features of the Immune System which Relate to the Area-Code Hypothesis

Area-code molecules. The immune system is an intriguing microcosm of the differentiating organism. Lymphocytes develop along two separate cell lineages -- B cells and T cells. Cell-surface molecules are expressed at various developmental stages of these lineages. Area-code molecules confer upon lymphocytes specific cell-surface addresses that direct two important recognition phenomena -- cell-cell interactions and migration to specific tissues. The antibody molecule, a prototype area-code molecule, is divided into discrete molecular domains which carry out distinct recognition and effector functions. The immune system employs combinatorial mechanisms at the genetic, evolutionary, molecular and supramolecular levels to amplify information.

DNA modification. The commitment of a lymphocyte to express a particular antibody polypeptide appears to require the DNA translocation of distinct V and C genes. This somatic modification of chromosomes can be passed on to progeny in a stable and heritable fashion. The DNA translocation even may create new structural genes and also alter the organization of control elements -- thus modifying the future developmental options of a particular cell lineage.

Multigene families. Antibody molecules are coded for by three multigene families. Multigene families are a fundamental unit of chromosomal organization and evolution in the immune system and presumably in other complex eukaryotic systems.

Let us now consider the area-code hypothesis in more general terms.

A. The Area-Code Hypothesis Suggests that Cell-Surface Recognition Molecules Form a Recognition Code on the Cell Surface

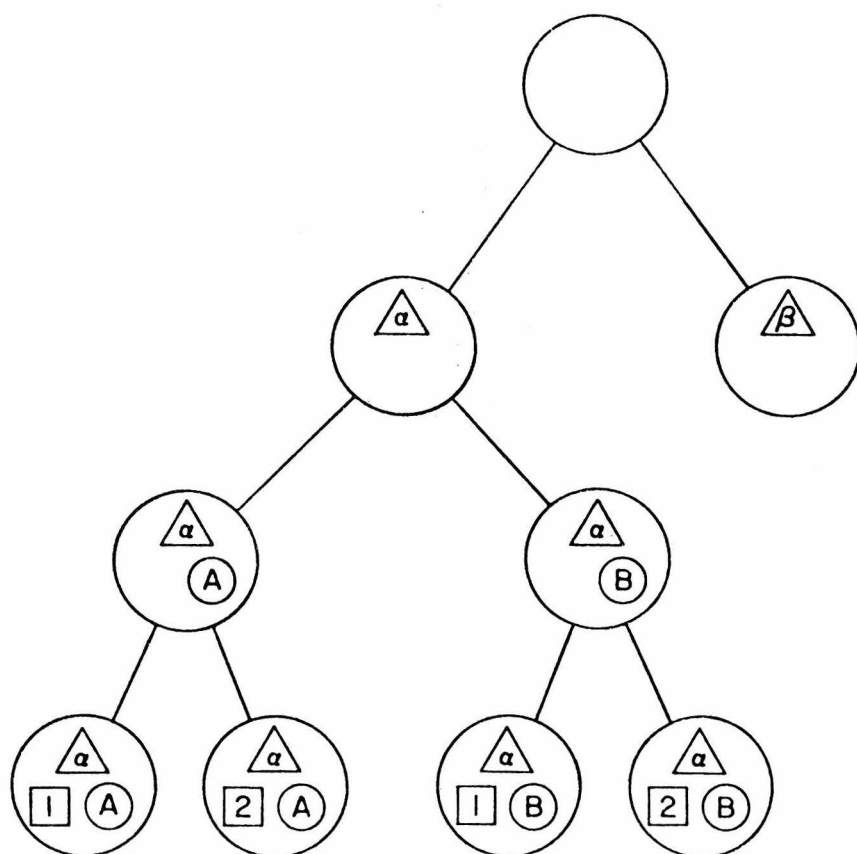
General. As a cell undergoes successive stages of differentiation, changing patterns of area-code molecules are displayed on the cell surface (Fig. 11). These molecules, singly or in groups, constitute a cell-surface display system that gives a cell or a group of cells an individual address much as the collective digits in a phone number or postal zip code identify individual locations. Area-code molecules expressed early in the differentiation of cell lineages, if not lost, will constitute a portion of the cell-surface address that identifies those cell lineages with an earlier embryonic origin than other area-code molecules expressed late in differentiation. For example, the α molecules expressed early in the differentiation of the hypothetical cells in Fig. 12 identify earlier lineage relationships than do the $\underline{A}$ molecules expressed somewhat later. Thus one can understand how the early digits in the area-code address specific general tissue relationships, whereas the later digits confer individual specificity (Fig. 12).

The area-code molecules which constitute these cell-surface displays may serve as molecular addresses to direct the migrating embryonic cells to appropriate locations or to permit specific cell-cell interactions. From Fig. 12 it can be seen how a cell which specifically recognizes, for example, the $\underline{\alpha}$, $\underline{1}$, $\underline{B}$ cell, could search and find that cell. The searching process may be a random one, or it may take advantage of the adjacent cells with slightly different area codes. For example, the cell could first find $\underline{\alpha}$, $\underline{10}$, $\underline{B}$, then $\underline{\alpha}$, $\underline{9}$, $\underline{B}$, and so on up the "cell-surface gradient" until

Figure 11. The model of the display of area-code molecules. For the sake of simplicity the possibilities of loss of area-code molecules, simultaneous appearance of two or more area-code molecules, sharing of area-code molecules, etc., are not indicated. The use of the T lymphocyte lineage is for illustration purposes only.

CELL	SURFACE	AREA CODE
Zygote	)	
Pre-Lymphocyte	) [▽]	1
Pre-T Lymphocyte	) [▽] _○	1 3
Thymocyte	) [▽] _{○ □}	1 3 6
Peripheral T Cell	) [▽] _{○ □ ○}	1 3 6 7

Figure 12. A model of the lineage of a differentiation cell line. α , β , A, B, 1, and 2 denote area-code molecules.



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it contacts α , 1, B. Thus a cell's search might be towards an increasingly better match between its area-code molecules and those of the target cell. If it strays off the path, to, e.g., α , 8, A, then the matching would decrease, and the cell could act accordingly by returning to the appropriate cell-surface gradient. Once at the target site, with its area-code maximally matched, the cell would lose its motility, perhaps through a process analogous to contact inhibition. Thus cell-surface as well as diffusible molecule gradients may play an important role in development.

Diverse cell-surface addresses may be generated by the various mechanisms employed by the immune system (Table III). These include genetic, protein and cell-surface display strategies. These mechanisms are capable of generating an enormous array of distinct cell-surface addresses from relatively few germ-line genes. Cells may interact with one another via area-code molecules by one of two general mechanisms -- self-self recognition or lock-and-key recognition (Fig. 13). Thus area-code molecules play a fundamental role in the highly precise cell-cell interactions of embryogenesis.

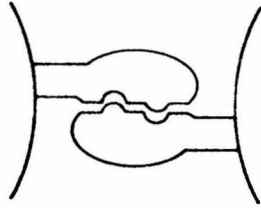
B. DNA Modification may be a Fundamental Mechanism for Cellular Differentiation

The generality of DNA modification in somatic cell lines. In addition to the translocation of V and C genes, there is evidence for DNA modification during differentiation in a variety of developmental systems. For example, chromosomal diminution or loss occurs in organisms as diverse as dipteran insects (43), ascarid nematodes (55), and copepodes (56). In these cases, somatic cells discard much of the DNA carried by germ-line

Figure 13. Models for specific cell-cell interactions.

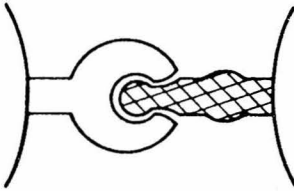
a) Self-self. b) Lock-and-key.

A.



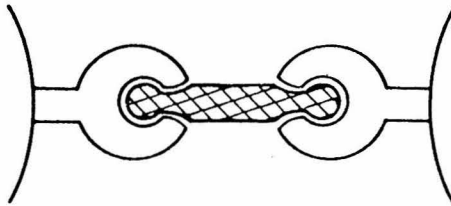
SELF-SELF

B.
i.



LOCK and KEY

ii.



LOCK and KEY

cells. This loss is due to the fragmentation or reduction in size of chromosomes when somatic cell lineages diverge from the germ line. In addition, various types of chromosomal modifications are seen in the somatic cells of a variety of organisms such as maize (57,58), snapdragons (59), and Drosophila (60). These genetic events, apparently due to the insertion and excision of DNA elements, include the turning on and off of genes, and the transposition, deletion, and inversion of chromosomal segments. Some of these DNA modifications appear to occur in a more or less programmed fashion while others are viewed as genetic instabilities leading to random genetic changes during development. Some of these phenomena appear very similar to those exhibited by the insertion sequences of prokaryotes (61). Insertion sequences are specific DNA sequences which insert into and excise from bacterial chromosomes presumably by crossing over (62). Indeed, the alternative expression of two genes in Salmonella for flagellar proteins is mediated by DNA inversions (63). Accordingly, it appears that DNA modifications may play a role in gene expression in a wide variety of systems. Admittedly few examples have been described for eukaryotes, but the search for DNA modification during somatic differentiation is an extremely active area of research (64). Our feeling is that DNA modification will be a fundamental mechanism of gene expression in simple as well as complex gene systems (65,66).

DNA modification and regulatory elements. B cells never express a V gene or a C gene unless they have been joined. Thus the translocation process creates a complete structural gene as well as activating (and rearranging) the regulatory elements necessary for its expression. So far the modification or rearrangement of structural genes has been found only in the immune system. However, the modification or rearrangement

of regulatory elements may be more common. Single genes can be turned on or off by appropriate modification of its regulatory element, as seen with the Salmonella flagellar proteins (63). In addition, a whole gene complex may be activated by the modification of a regulatory element. For example, a reversible inversion of a promoter element has been postulated for the alternate and mutually exclusive expression of two closely linked gene complexes which determine alternate mating types in the fission yeast (67). If one of the activated genes has a regulatory product (e.g., an inducer), then genes controlled by that product would be affected, even if they are on another part of the genome. This appears to be the case with the Spm system in maize (58). In general, whole batteries of genes, linked or unlinked, could be controlled by the DNA modification of control elements. Thus area-code systems may regulate gene expression through the DNA modification of control elements.

Do the nuclear transplantation experiments of Gurdon and others argue against DNA modification as a general mechanism of differentiation?

It is possible to transplant a somatic nucleus into an enucleated egg and then to stimulate this chimeric cell to begin embryogenesis (68,69). When nuclei from blastula cells are transplanted to enucleated eggs, a high fraction of the eggs develop normally to form fertile, adult frogs. When nuclei from the intestinal cells of young embryos are used, most become defective embryos but a very small number produced fertile, adult frogs (70). These experiments suggest to some that differentiation is a reversible process and therefore the DNA in all somatic cells is identical to that of the germ cells. However, an important reservation about these experiments

should be noted. There are striking differences in the results obtained with blastula and somatic cell nuclei. Clearly these nuclei have quite different potentials for generating adult frogs. Perhaps germ cells (or early stem cells), which are known to migrate through the intestinal mucosa, are the source of the nuclei which produce the few fertile adults in the nuclear transplantation experiments carried out with putative intestinal nuclei. To rule out this possibility, nuclei from lymphocytes and differentiated epidermal cells of adults have been transplanted into enucleated eggs (71,72). In these cases, no fertile adult frogs are obtained. All embryos died at or before the early tadpole stage. Indeed, the nuclei from an aneuploid liver cell line from a frog supports development almost as well as the adult somatic cell nuclei (73). These results are not inconsistent with the proposition that DNA modifications occur as somatic cell lineages undergo development. Perhaps somatic nuclei support development in nuclear transplantation experiments up until the stage at which the genes which have been modified are needed.

We feel that the nuclear transplantation experiments do not rule out the possibility that DNA modifications occur as a general phenomena in the differentiation of somatic cells. Certainly the experiments already discussed in relation to the development of the immune system provide compelling evidence for DNA modification in lymphocytes. The evidence for DNA modifications is also compelling in a variety of other systems as discussed above.

C. Multigene Families Appear to be a Fundamental Unit of Eukaryotic Gene Organization and Evolution

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General. Multigene families display four fundamental characteristics: multiplicity, close linkage, sequence homology and similar or overlapping functions (see [74] for review). A variety of eukaryotic genes exhibit these properties including the ribosomal RNA genes (75), tRNA genes (76), histone genes (77), the β -like globin genes (78) and the DNA satellites (79). Multigene families may range in size from a few gene members to thousands of gene members. Indeed, simple multigene families appear to code for several of the differentiation antigens found on lymphocytes including TL (80), Qa (81) and Ia (82). Accordingly, area-code families may range from few to many gene members.

Evolution of primordial multigene families. Multigene families are found in eukaryotes, but not in prokaryotes (74). Multigene families may have evolved in response to the informational requirements of differentiation in multicellular organisms (see Fig. 1). This supposition suggests that multigene families will be employed in cell-recognition systems and, accordingly, will code for a variety of area-code molecules.

New evolutionary unit. New multigene families can arise in two ways: 1) gene duplication from a single gene, or 2) duplication of all or part of a preexisting multigene family. The duplication of a preexisting multigene family may occur in two ways: tetraploidization (see [83]) or duplications and translocation of a portion of a chromosome. It obviously requires a long period of evolutionary time to produce a large multigene family starting with a single gene. In contrast, the duplication of an entire multigene family presumably generates new gene families by a single genetic event. Thus

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evolution can proceed at a more rapid pace by the instantaneous generation of entire families of new genes. The duplication of multigene families is illustrated by the homology relationships of the antibody gene families which suggest that all three gene families were derived from a common ancestral multigene family. Thus the multigene family is a basic unit of eukaryotic evolution. New and complex area-code gene families can obviously be created rapidly.

Homologies among multigene families. A new multigene family arising by duplication from a primordial gene family may interact with its sister-gene family as occurs between the light and heavy chain gene families of antibodies. Alternatively, it may evolve to assume new functions. Evolutionary relationships among multigene families might be discerned from two aspects of homology: i) amino acid sequence homology among gene products and ii) shared complex control mechanisms such as V-C translocation. Once more data are available on a variety of area code systems, these homology analyses should allow us to construct a genealogical tree of relationships among the multigene families encoding these area-code systems.

POTENTIAL SYSTEMS EMPLOYING THE AREA-CODE STRATEGY

A. The Immune System

Antibodies. Antibody molecules and genes provide a model system for posing questions about other complex area-code systems (Table IV). Other area-code systems may employ some but not necessarily all of the strategies of the antibody system. Indeed, some will probably present novel strategies and characteristics. However, the questions posed in Table IV help us to approach experimentally other area-code systems, both simple

Table IV. Questions about Potential Area-Code Molecules and
Genes based on an Analysis of the Immune System

A. Protein Level

1. Are the molecules composed of multiple subunits?
2. If so, do they exhibit combinatorial association?
3. Are homology units present? How large are they?
4. Do they show homology to immunoglobulins? To other known gene systems?
5. Are V and C regions present?
6. What type of molecular recognition functions do they perform?

B. Genetic Level

1. Are the polypeptides encoded by multigene families?
2. Are V and C genes present?
3. How large is the gene family?
4. How are the various control and structural genes organized?

C. Regulatory Level

1. Is there V-C translocation?
2. What is the pattern of clonal expression of individual area-code molecules?
3. What is the nature of the programs which govern development?

D. Evolutionary Level

1. Are the multigene families within the system homologous to one another?
 2. Are they related to other known multigene systems?
-

and complex, at the protein, genetic, regulatory, and the evolutionary levels.

The study of antibody molecules and genes is currently under intensive investigation in many different laboratories (see [84]). Important unresolved experimental questions include the following: i) What are the mechanisms responsible for antibody diversity? ii) What is the molecular mechanism of V-C translocation? iii) How are antibody genes and their control elements organized on vertebrate chromosomes? iv) How many V genes are present in various antibody gene families? v) Are V genes translocated to C genes according to an orderly developmental program? vi) What developmental strategy is used for programming specific combinations of light chain genes and heavy chain genes? Certainly the investigation of these and many other questions will provide important insights into this model area-code system.

T-cell receptors. The T-cell receptor has been an elusive entity. Recently, however, serological and genetical studies suggest that the T-cell receptor for antigen binding employs a V_H gene from the B-cell H chain family presumably associated with a new C_H gene (see 85). The presence of a light chain in the T-cell receptor is still a matter of uncertainty (86). Thus T and B cells both employ the same V_H gene family. However, the expression of V_H genes on T or B cells appears to be controlled by the nature of C_H gene to which they are translocated. The various C_H genes given in Fig. 7 are expressed on B cells, whereas the putative C_H gene(s) for T cells is expressed only on that cell lineage. Thus the same area-code gene library can be employed for two distinct receptor systems by

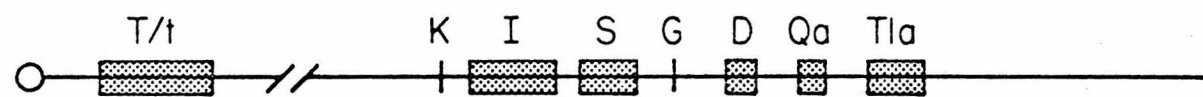
virtue of the DNA translocation mechanism and the differential expression of C_H genes in the T- and B-cell lineages.

The study of T-cell receptors is in its infancy. Very little is known about the structure of these molecules. All of the questions raised for antibody molecules and genes of B cells can be asked of the T-cell receptor (see above and Table IV).

H-2 complex. Chromosome 17 of the mouse appears to code for a series of multigene families (87) (Fig. 14). For example, by simplistic genetic calculations the region between K and Tla has sufficient DNA to code for about 16,000 polypeptides 100 residues in length (e.g., V region size). The T/t family with at least six complementation groups appears to encode cell-surface proteins regulating neuroectodermal development during embryogenesis (1). The S family codes for the structural and/or regulatory elements of several complement components (88). The Qa region appears to have two or more genes coding for differentiation antigens on lymphocytes (89). The D (90), I (87), and Tla (91) gene families also appear to have two or more homologous gene members. Moreover, indirect evidence suggests that the genes coding for the transplantation antigens may be present in multiple copies (92). Accordingly, chromosome 17 of the mouse appears to be a library of gene families coding for cell-surface molecules.

The gene products of the K, D, Qa, and Tla gene families show two interesting relationships to one another. i) All are cell-surface glycoproteins of about 45,000 molecular weight (89,93). ii) Each is associated with β_2 -microglobulin, a polypeptide of about 100 residues that demonstrates significant homology with homology units of antibody constant regions (94,95). These relationships suggest that these differentiation antigens may be homologous

Figure 14. Chromosome 17 of the mouse. Shaded bars represent two or more closely linked homologous genes.



Chromosome 17

and thereby share a common evolutionary origin. This supposition is supported by preliminary amino acid sequence analyses of the N-terminal regions of the K and D molecules that demonstrates sequence homology. A similar analysis has not yet been carried out on the Qa and Tla gene products. The association between these differentiation antigens and β_2 -microglobulin provides a fascinating relationship between the antibody system and these cell-surface molecules. Indeed, amino acid sequence data have demonstrated a striking homology between an internal 17-residue portion of a human transplantation antigen and a portion of the antibody V region (C. Terhorst and J. Strominger, personal communication). If further sequence analyses confirm this observation, it appears that antibodies and transplantation antigens may be evolutionarily related. It will be interesting to determine whether the same is true of the Qa and Tla gene products.

The functions of these cell-surface molecules are uncertain, but the Qa, Tla and the transplantation antigens are expressed in different patterns on different populations of lymphocytes. Thus these cell-surface molecules may be encoded by small multigene families that diverged from a common ancestor and which have diverged to carry out distinct cell-surface functions.

One gene product from the T/t system has been characterized and it also appears to be 45,000 in molecular weight and associated with a β_2 -microglobulin-like molecule (96). The I region molecules differ in molecular weight from the transplantation antigens and do not show obvious sequence homology at their N-termini (97). Preliminary amino acid sequence studies indicate that the non-membrane-associated S region gene products also fail to show homology to immunoglobulins (98). Clearly additional data are necessary before any convincing relationships can be established among these gene families.

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Certain of these differentiation antigens carry out cell-surface recognition functions (e.g., K, Ia, and D) and, accordingly, are area code molecules. The others may be area-code molecules, although their functions are generally unknown. We are in a position to ask of these gene families many of the questions listed in Table IV.

B. Other Developmental Systems

General. There are a variety of developmental systems where area-code molecules appear to play an important role. For example, embryonic tissues appear to have cell-surface recognition molecules that mediate tissue-specific cellular interactions (99). The imaginal disks of Drosophila larva can be disassociated and reaggregated in a manner that suggests area-code molecules may be involved (100). The retinal nerves of the goldfish upon sectioning appear capable of reattaching to their specific tectal counterparts (101). One possibility is that these specific interactions are mediated through the cell-surface addresses provided by area-code molecules. The difficulty with each of these systems has been that large quantities of homogeneous area-code molecules are difficult to obtain. A promising approach is to create homogeneous cell lines from individual cells in these systems. Homogeneous cell lines could be produced from cells transformed by viral, chemical, or physical agents. In addition, permanent cell lines may be generated by fusing primary cells with established cell lines. This latter approach has been successful in creating cell lines secreting homogeneous anti-H2 antibodies by fusing normal B cells synthesizing anti-H-2 antibodies with B cell tumors (102).

The production of large quantities of cells from homogeneous cell lines appears to be one of the primary prerequisites for the detailed biochemical analysis of many interesting developmental systems.

Tumor specific antigens. Certain tumor specific antigen systems appear to be potential area-code systems which can be studied currently. One of the key experimental approaches to the study of the immune system was the use of tumors cultured in vivo and in vitro. These tumors were homogeneous clones of lymphoid cells and provided large amounts of experimental material. Since we believe that different embryonic cells have different cell-surface arrays of area-code molecules, we might ask if there are presently available tumor cell lines which display a large diversity of line-specific surface molecules or antigens. In fact, such individually specific cell-surface antigens have been found on a wide variety of tumors induced by physical or chemical agents. In certain inbred strains of mice both methylcholanthrene (103) and ultraviolet light (104) induce tumors with individually unique tumor antigens. The repertoire of these unique tumor antigens appears very large (103). Moreover, large quantities of tumor cells can be raised for analysis of the tumor antigens and their genes. It will be important to determine whether these antigens are abnormal molecules related only to tumor transformation or whether they might indeed be area-code molecules playing a fundamental role in some stage of development. For these systems it will be interesting to answer many of the questions raised in Table IV.

CODA

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An analysis of the immune system has led to the area-code hypothesis. The strategies employed by the antibody system for gene organization, regulation and evolution provide a model for asking experimentally testable questions about other potential area-code systems (Table IV). Investigations of area-code systems have two basic requirements. First, homogeneous clones of cells expressing the potential area-code molecules must be available. Second, microchemical techniques must be employed to characterize these molecules which are generally available in very small quantities. Our laboratories have been engaged in developing microsequencing techniques for the last several years. We are now applying these techniques to the analysis of several potential area-code systems (93,97) including the ultraviolet light-induced tumors of C3H mice. This tumor system may provide a second detailed view of a complex area-code system.

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FOOTNOTE

¹The nucleic acid studies on antibody genes provide unequivocal evidence for two suppositions. First, the DNA sequence data on the embryonic V gene (43) provide compelling evidence that the V and C genes are distinct in the germ line. Second, the restriction enzyme studies on embryonic and differentiated DNA (44,45) argue that a DNA modification event has occurred during lymphocyte differentiation. This DNA modification could be anything which alters the restriction enzyme sites in the adult DNA with respect to embryonic DNA. The obvious DNA modification to explain these restriction enzyme patterns would be a translocation of the V and C gene sequences (Fig. 7). Other DNA modifications that do not rearrange V and C gene sequences require two assumptions. i) DNA modification such as base methylation changes the restriction enzyme sites. ii) The separate V and C genes are transcribed as a single mRNA. In this regard, recent observations suggest that a single mRNA can be transcribed from separate DNA segments on the adenovirus chromosome (105,106). These alternative possibilities for DNA modification can be tested by examining the organization of V and C genes in myeloma DNA. However, we tend to favor the simpler hypothesis of DNA translocation and assume throughout this paper that the DNA modification event in lymphocyte differentiation is a joining of the V and C genes.

REFERENCES

1. Bennett, D.: Cell 6:441 (1975).
2. Changeux, J.-P., and Danchin, A.: Nature 264:705 (1976).
3. Sperry, R. W.: Proc. Nat. Acad. Sci. USA 50:703 (1963).
4. Cantor, H., and Boyse, E. A.: Immunol. Rev. 33:105 (1977).
5. Jerne, N. K.: Sci. Amer. 229:52 (1973).
6. Metcalf, D., and Moore, M. A. S.: in "Haemopoietic Cells," A. Neuberger, and E. L. Tatum (Eds.), North Holland Research Monographs, Amsterdam, Front. Biol. 24: 172 (1971).
7. Cantor, H., and Weismann, I.: Progress in Allergy 20:1 (1976).
8. Houssaint, E., Belo, M., and Le Douarin, N. M.: Dev. Biol. 53:250 (1976).
9. Good, R. A.: The Harvey Lectures. New York and London: Academic Press, series 67:1 (1973).
10. Cazenave, P.-A., Cavaillon, J. M., and Bona, C.: Immunol. Rev. 34:34 (1977).
11. Boyse, E. A., and Old, L. J.: Ann. Rev. Genet. 3:269 (1969).
12. Storrie, B., Goldstein, C., Boyse, E. A., and Hammerling, U.: J. Immunol. 116:1358 (1976).
13. Klein, J.: "Biology of the Mouse Histocompatibility-2 Complex." New York: Springer-Verlag. 135 (1975).
14. Takahashi, T., Olds, J., and Boyse, E. A.: J. Exp. Med. 131:1325 (1970).
15. Raff, M.: Nature 242:19 (1973).
16. Jerne, N. K.: Ann. Immunol. (Inst. Pasteur) 125C:373 (1974).

209

17. Jerne, N. K.: The Harvey Lectures. New York and London: Academic Press, Series 70:93 (1976).
18. Jerne, N. K.: 27th Mosbacher Colloquium. Berlin: Springer-Verlag, 259 (1976).
19. Cantor, H., Shen, F. W., and Boyse, E. A.: J. Exp. Med. 143:1391 (1976).
20. Shevach, E. M.: Fed. Proc. 35:2048 (1976).
21. Metcalf, D., and Moore, M. A. S.: in "Hemopoietic Cells," A. Neuberger and E. L. Tatum (Eds.), North Holland Research Monographs, Amsterdam, Front. Biol. 24:70 (1971).
22. Katz, D. H., and Benacerraf, B., Eds. "The Role of Products of the Histocompatibility Complex in Immune Responses." Academic Press, New York (1976).
23. Doherty, P. C., Götze, D., Trinchieri, G., and Zinkernagel, R. M.: Immunogenetics 3:517 (1976).
24. Möller, G. (Ed.) "Biochemistry and Biology of Ia Antigens." Immunol. Rev. 30 (1976).
25. Klein, J.: Ann. Rev. Genet. 8:63 (1974).
26. Rosenthal, A. S., and Shevach, E. M.: J. Exp. Med. 138:1194 (1973).
27. Cosenza, H., Julius, M. H., and Augustin, A. A.: Immunol. Rev. 34:3 (1977).
28. Rodkey, L. S.: J. Exp. Med. 139:712 (1974).
29. Eichmann, K., and Rajewsky, K.: Eur. J. Immunol. 5:661 (1975).
30. Cantor, H.: in "The Immune System II. Regulatory Genetics," L. Herzenberg and E. Sercarz (Eds.), Academic Press, New York, in press (1977).

210

31. Boyse, E. A., and Cantor, H.: in "1st International Conference on the Molecular Basis of Cell-Cell Interaction" (San Diego): Academic Press, New York, in press (1977).
32. Huber, B., Cantor, H., Shen, F. W., and Boyse, E. A.: J. Exp. Med. 144:1123 (1976).
33. Gally, J.: in "The Antigens" M. Sela (Ed.) Academic Press, New York and London, I:162 (1973).
34. Gally, J., and Edelman, G. M.: Ann. Rev. Genet. 6:1 (1972).
35. Edelman, G. M., Cunningham, B. A., Gall, W., Gottlieb, P., Rutishauser, U., and Waxdal, M.: Proc. Nat. Acad. Sci. USA 63:78 (1969).
36. Kehoe, J. M., and Fougereau, M.: Nature 224:1212 (1969).
37. Mage, R., Lieberman, R., Potter, M., and Terry, W. D.: in "The Antigens" M. Sela (Ed.) Academic Press, New York and London, I:300 (1973).
38. Hood, L., Kronenberg, M., Early, P., and Johnson, N.: in "The Immune System II. Regulatory Genetics," L. Herzenberg and E. Sercarz (Eds.), Academic Press, New York, in press (1977).
39. Hood, L., Loh, E., Hubert, J., Barstad, P., Eaton, B., Early, P., Fuhrman, J., Johnson, N., Kronenberg, M., and Schilling, J.: Cold Spring Harb. Symp. Quant. Biol. 41:817 (1977).
40. Hood, L.: Fed. Proc. 31:177 (1972).
41. Hozumi, N., and Tonegawa, S.: Proc. Nat. Acad. Sci. USA 73:3632 (1976).
42. Tonegawa, S., Hozumi, N., Matthysens, G., and Schuller, R.: Cold Spring Harbor Symp. Quant. Biol. 41:877 (1977).
43. Tonegawa, S., Brack, C., Hirama, M., Hozumi, N., and Matthysens, G.: Cold Spr. Harb. Symp. Quant. Biol. 42, in press (1977).

211

44. Dreyer, W. J., Gray, W., and Hood, L.: Cold Spr. Harb. Symp. Quant. Biol. 32:353 (1967).
45. Edelman, G. M.: Cold Spr. Harb. Symp. Quant. Biol. 41:891 (1977).
46. Klinman, N. R., and Press, J. L.: J. Exp. Med. 141:1133 (1975).
47. Sigal, H. H., Gearhart, P. J., Press, J. L., and Klinman, N. R.: Nature 259:51 (1976).
48. Lydyard, P. M., Grossi, C. E., and Cooper, M. D.: J. Exp. Med. 144:79 (1976).
49. Amzel, L., Poljak, R., Saul, F., Varga, J., and Richards, F.: Proc. Nat. Acad. Sci. USA 71:1427 (1974).
50. Padlan, E. A., Segal, D. M., Cohen, G. H., and Danes, D. R.: in "The Immune System: Genes, Receptors, Signals," E. Sercarz, A. Williamson, and C. Fox (Eds.), Academic Press, New York and London, 7 (1974).
51. Edmunson, A. B., Ely, K. R., Girling, R. L., Abola, E. F., Schiffer, M., Westholm, F. A., Fausch, N. D., and Deutsch, H. F.: Biochemistry 13:3816 (1974).
52. Cohn, M., Blomberg, B., Geckeler, W., Raschke, W., Riblet, R., and Weigert, M.: in "The Immune System: Genes, Receptors, Signals," E. Sercarz, A. Williamson, and C. Fox (Eds.), Academic Press, New York and London, 89 (1974).
53. Tonegawa, S.: Proc. Nat. Acad. Sci. USA. 73:203 (1976).
54. Bantock, C. R.: J. Embryol. Exp. Morph. 24:257 (1970).
55. Goldstein, P.: J. Morph. 152:141 (1977).
56. Beermann, S.: Chromosoma (Berl.) 60:297 (1977).
57. McClintock, B.: Brookhaven Symp. Biol. 18:162 (1965).
58. Nevers, P., and Saedler, H.: Nature (Lond.) 268:109 (1977).

212

59. Fincham, J. R. S., and Harrison, B. J.: *Heredity* 22:211 (1967).
60. Green, N. M.: *Genetics* 73 (suppl):187 (1974).
61. Starlinger, P.: *Critical Rev. Microbiol.*, in press. (1977).
62. Cohen, S. N.: *Nature* 263:731 (1976).
63. Zieg, J., Silverman, M., Hilmen, M., and Siman, M.: *Science* 196:170 (1977).
64. Bukhari, A. J. (Ed.), "DNA insertion elements, plasmids and episomes," Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, in press (1977).
65. Holliday, R., and Pugh, J. E.: *Science* 187:226 (1975).
66. Sager, R., and Kitchin, R.: *Science* 189:426 (1975).
67. Egel, R.: *Nature (Lond.)* 266:172 (1977).
68. Gurdon, J. B., and Laskey, R. A.: *J. Embryol. Exp. Morph.* 24:227 (1970).
69. Briggs, R., and King, T. F.: *Proc. Nat. Acad. Sci. USA* 38:455 (1972).
70. Gurdon, J. B., and Uehlinger, V.: *Nature* 210:1240 (1966).
71. Gurdon, J. B., Laskey, R. A., and Reeves, O. R.: *J. Embryol. Exp. Morph.* 34:93 (1975).
72. Wabl, M. R., Brun, R. B., and DuPasquier, L.: *Science* 190:1310 (1975).
73. Kobel, H. R., Brun, R. B., and Rischberg, M.: *J. Embryol. Exp. Morph.* 29:539 (1973).
74. Hood, L., Campbell, J. H., and Elgin, S. C. R.: *Ann. Rev. Genet.* 9:305 (1975).
75. Birnstiel, M. L., Chipchase, M., and Spiers, J.: *Prog. Nucleic Acid Res. Mol. Biol.* 11:351 (1971).
76. Clarkson, S. G., and Birnstiel, M. L.: *Cold Spring Harb. Symp. Quant. Biol.* 38:451 (1973).

213

77. Schaffner, W., Gross, K., Telford, J., and Birnstiel, M.: *Cell* 8:471 (1976).
78. Kitchen, H., and Bayer, S. (Eds.), "Hemoglobin: Comparative Molecular Biology Models for the Study of Disease." *Ann. N.Y. Acad. Sci.* 24 (1974).
79. Gall, J. G., Cohen, E. H., and Atherton, D. D.: *Cold Spring Harb. Symp. Quant. Biol.* 38:417 (1973).
80. Boyse, E. A., Stockert, E., and Olds, L. J.: in "International Convocation on Immunology", Rose, N. R., and Milgrom, F. (Eds.), S. Karger, Basel, 353 (1968).
81. Flaherty, L.: *Immunogenetics* 3:533 (1976).
82. McDevitt, H. O., Delovitch, T. L., Press, J. L., and Murphy, D. B.: *Transplant Rev.* 30:197 (1976).
83. Ohno, S.: "Evolution by Gene Duplication", Springer, New York, (1970).
84. "Origins of Lymphocyte Diversity." *Cold Spring Harb. Symp. Quant. Biol.* 41:627 (1977).
85. Janeway, C. A., Wigzell, H., and Binz, H.: *Scand. J. Immunol.* 5:993 (1976).
86. Rajewsky, K.: in "The Immune System II. Regulatory Genetics," L. Herzenberg and E. Sercarz (Eds.), Academic Press, New York, in press (1977).
87. McDevitt, H. O.: *Fed. Proc.* 35:2168 (1976).
88. Schreffler, D. C.: *Transplant. Rev.* 32:140 (1976).
89. Michaelson, J., Flaherty, L., Vitetta, E., and Poulik, M. D.: *J. Exp. Med.* 145:1066 (1977).
90. Hansen, T. H., Cullen, S. E., and Sachs, D. H.: *J. Exp. Med.* 145: 438 (1977).

214

91. Klein, J.: "Biology of the Mouse Histocompatibility-2 Complex,"
Springer-Verlag, New York, 241 (1975).
92. Garrido, F., Festenstein, H., Schirmacher, V.: *Nature* 261:705
(1976).
93. Silver, J., and Hood, L.: in "Contemporary Topics in Immunobiology,"
H. M. Eisen and R. A. Reisfeld (Eds.), Plenum Publ., NY, 5:35 (1976).
94. Smithies, O., and Poulik, M. D.: *Proc. Nat. Acad. Sci. USA* 69:2914
(1972).
95. Cunningham, B. A., Wang, J. L., Berggård, I., and Peterson, P. A.:
Biochemistry 12:4811 (1973).
96. Jacob, F.: *Immunol. Rev.* 33:3 (1977).
97. Silver, J., Cecka, J. M., McMillan, M., and Hood, L.: *Cold Spring
Harb. Symp. Quant. Biol.* 41:369 (1977).
98. Bolotin, C., Morris, S., Tack, B., and Prahl, J.: *Biochemistry*
16:2008 (1977).
99. Moscona, A. A., and Housman, R. E.: in "Cell and Tissue Interactions,"
J. Lash and M. Burger (Eds.), Raven Press, New York, in press (1977).
100. Garcia-Bellido, A.: "Results and Problems in Cell Differentiation,"
H. Ursprung, and R. Nöthiger, R. (Eds.), Springer-Verlag, Berlin,
5:59 (1972).
101. Sperry, R. W.: "Organogenesis," R. L. DeHaan and H. Ursprung (Eds.),
Holt, Reinhart, and Winston, Inc., New York, 161 (1965).
102. Galfre, A., Howe, S. C., Milstein, C., Butcher, A. W., and Howard, J. C.:
Nature (Lond.) 266:550 (1976).
103. Basombrio, M. A.: *Cancer Res.* 30:2458 (1970).
104. Kripke, M. L.: *J. Nat. Cancer Inst.* 53:1333 (1974).
105. Berget, S. M., Berk, A. J., Harrison, T., and Sharp, P.: *Cold Spring
Harb. Symp. Quant. Biol.* 42, in press (1977).

106. Broker, T. R., Chew, L. T., Dunn, A., Gelinas, R. E., Hassell, J.,
Kleorig, D. F., Lewis, J., Roberts, R. J.: Cold Spring Harb. Symp.
Quant. Biol. 42, in press (1977).