STUDIES ON THE MAJOR HISTOCOMPATIBILITY COMPLEX OF THE MOUSE AND RAT

Thesis by

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To BFBF

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Abstract

This thesis contains investigations into the Major Histocompatibility Complex (MHC) of two closely related species, rats and mice. In particular, I have concentrated on molecules encoded in one part of the MHC, the I region in mice, and its equivalent in rats. The first part of the thesis consists of experiments dealing with the expression of molecules encoded by genes in the I region, the Ia molecules. Ia molecules are expressed on immune related cells and surprisingly on epidermal cells.

Several conclusions can be drawn from my studies on these epidermal Ia molecules. The Ia molecules isolated from radiolabeled detergent solubilized epidermal cell extracts are not contributed by contaminating lymphocytes. The Ia molecules from epidermal cell extracts are identical to their counterparts isolated from spleen cells by both sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and high pressure liquid chromatography tryptic peptide map analyses. The Ia molecules are synthesized by a non-T and non-B cell bone-marrow-derived cell. This cell is probably the macrophage-like Langerhans cell. This work supports the theory that Ia molecules are involved in the immune response and are present only on immune related cells.

The second part of this thesis deals with the Class II (Ia-like) molecules encoded by the rat equivalent of the I region. Two Class II molecules can be immunoprecipitated using cross-reactive mouse anti-Ia sera. These reagents will be extremely useful in the further elucidation of the rat MHC.

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INTRODUCTION

The major histocompatibility complex (MHC) of vertebrates is comprised of a cluster of tightly linked loci which regulate many aspects of the immune response. It is present in a wide variety of species. The following systems have been described: H-2 (mouse), HLA (man), RhL-A (rhesus monkey), ChL-A (chimpanzee), RT1, AgB or H-1 (rat), DL-A (dog), SL-A (pig), GPL-A (guinea pig), XL-A (Xenopus), B (chicken), RbH-1 or RL-A (rabbit) and BL-A (cattle). Obviously there is no consistent nomenclature for the MHCs of different species! The MHC of all species have not been equally well characterized; the best studied are those of mice and man. The MHC of different species is involved in several immune related reactions. They include: 1) T cell activation and differentiation, 2) graft rejection, 3) regulation of cellular responses as well as humoral responses, and 4) control of some complement components. A further prominent feature in the MHC of mice and of man is the striking polymorphism of several MHC proteins. Exactly how this relates to their function is unknown. I shall briefly summarize what is known about the MHC of rats and mice.

Mouse MHC

The pioneer work of Peter Gorer over forty years ago suggested that the product of a single locus detected by hemagglutination also elicits rapid graft rejection (1). Because of the allograft rejection properties, Gorer's antigen II was designated Histocompatibility-2 or H-2. Gorer's finding was the basis for contemporary theories of transplantation, i.e., that genetically controlled antigens present in the graft but absent in the host elicit an immune response. Snell, using inbred strains originally established by Little, Strong, and others, constructed congenic resistant inbred lines by extensive backcrossing and selection. The importance of these congenic lines which differ only at genes of the H-2 complex, and genes closely linked to the H-2 complex, cannot be overemphasized. These

strains of mice, which he made freely available, gave investigators a tool to analyze the fine structure of the H-2 complex, its relevance to transplantation, cell-cell interactions, control of the immune response, susceptibility to disease, complement regulation, and immune cell differentiation (2).

It was shown that the H-2 locus was really a complex of genes. It could be subdivided into regions by recombination and one of the regions, the I region, was further divided into subregions. The distinction between a region and a subregion is largely historical and somewhat artificial. Although there are several designations for molecules encoded by the H-2 complex, one nomenclature which facilitates interspecies comparisons simply groups the molecules into classes I, II, or III (3). A summary of some of the characteristics of class I and II molecules is presented below. Class III molecules deal with complement components and will not be discussed. A review of class III molecules can be found elsewhere (4).

The class I molecules are heterodimers consisting of a 45,000 molecular weight glycoprotein subunit (K, D or L), and a 12,000 molecular weight subunit (β_2-microglobulin). The larger subunit is encoded in the H-2 complex. A moderate and ever-increasing amount of primary sequence is known ($\sim$60% of the residues of one class I molecule have been identified) (5). There are extensive serological and biochemical differences among alleles. Because the mouse K and D allelic gene products differ from one another by multiple amino acid residues and segregate in a Mendelian fashion, they have been termed complex allotypes (6). Further, there is no apparent K-ness or D-ness: that is, two K molecules are apparently no more closely related than a K and a D molecule (7). Class I molecules are extraordinarily polymorphic. Almost every wild mouse tested has a new K or D allele (8). The class I molecules are targets for graft rejection. They also function as the targets in the restricted killing of virally infected or TNP-modified cells (9, 10). They have a wide tissue distribution, being present on virtually every

cell type except adult germ cells and cells very early in development (2).

Class II molecules are encoded in the I region. The only gene products of the I region which have been identified by immunochemical techniques are the Ia antigens encoded by loci mapping to the I-A and I-E subregions (11, 12). The class II or Ia molecules are composed of at least two noncovalently associated glycoprotein subunits. There is an α subunit of approximately 34,000 molecular weight, and a β subunit of approximately 28,000 molecular weight. A third, apparently nonpolymorphic subunit called the constant or invariant spot has also been reported (13). In inbred strains of mice, there are extensive differences both serologically and biochemically among the α subunits and among the β subunits encoded by genes in the I-A subregion. There are fewer differences among alleles of the I-E subregion. In particular, the E_α molecule is highly conserved and is also highly homologous to its human and guinea pig counterparts (14, 15). Wild mice exhibit striking polymorphism of the I-A subregion products while the I-E subregion products are much less polymorphic (8). Genes in the I-A subregion either code for the E_β polypeptide or modify it (16). Further, not all inbred strains express molecules encoded in the I-E subregion. Genes controlling the humoral immune response to antigens map to both the I-A and I-E subregion (2). Class II molecules, or products of genes very closely linked to the I region, elicit graft rejection (17), T cell proliferation in mixed lymphocyte reactions (MLR), and seem to be necessary for interactions between macrophages and T cells (18). They are found on a variety of immunological factors which may function in cell-cell interaction (18). They are much more restricted in their tissue distribution than the class I molecules, being present only on immune related tissues, epidermal cells, and perhaps spermatozoa (8).

Rat MHC

The rat MHC, called variously the H-1, AgB or RT1 complex, is similar in many respects to the murine H-2 complex. Even though inbred rat strains were

initiated about the same time as inbred strains of mice, much less is known about the RT1 complex than the H-2 complex. It was not until the last few years that recombinants in the RT1 complex have been described and they still are not freely available. This has greatly hindered progress in the understanding of the RT1 complex. The complex has been divided into two regions, A and B. Serologically, the A region codes for at least one class I molecule and the B for at least one class II molecule (19, 20). The class I molecules immunoprecipitated by alloantisera are glycoproteins of approximately 45,000 molecular weight and are associated with β_2-microglobulin (21, 22). Partial N-terminal sequence analysis indicates that there are at least two class I molecules (21). The RT1.A alloantigens are the principal target for cytotoxic T cells. The alloantigens have a wide tissue distribution being expressed on lymphocytes, red blood cells, platelets and many other tissues (19, 23, 24). There are considerable differences among the class I molecules of inbred strains (25).

The class II molecules are composed of at least two polypeptides; an α of molecular weight 32,000 and a β of molecular weight 28,000 (22, 25). The α and β polypeptides are polymorphic. The class II molecules are restricted in their tissue distribution, being present on B lymphocytes and on an abundant, presumably nonlymphoid, cell of rat kidney (23, 27). The products of the RT1.B region (presumably class II molecules) stimulate various allogeneic responses in mixed lymphocyte reactions (MLR) (19, 28). Further, the control of the immune response to synthetic polypeptides maps to the RT1.B region (20). The most provocative feature of the rat MHC is its relative lack of polymorphism. When one traps wild rats, and screens them with a panel of antisera used to type inbred strains for class I molecules, almost all the wild rats react with one or two (but never three) of the sera (29). Thus it appears that the inbred strains express most of the polymorphism of the class I molecules of the entire species. Similarly, if one

types wild rats by their mixed lymphocyte reactions (MLR), which are thought to be a measure of the similarity of class II molecules, again the inbred strains of rats seem to contain most of the polymorphism of the entire species (30). This is quite different than the situation in mice and men.

Rationale

This thesis reports studies on proteins encoded by the MHC of rats (AgB or RT1) and mice (H-2). Many different cells must interact to generate and regulate an immune response. For example, macrophages must interact with both T cells and B cells to initiate a response, T cells must cooperate with B cells to stimulate them to become antibody-producing cells, and some T cells interact with other T cells to suppress an immune response. Ia molecules are intimately involved in all these processes. Thus, it was very puzzling to find Ia antigens expressed on epidermal cells. These cells do not have an obvious immune function. Was this finding the result of contaminating lymphocytes in the epidermal cell preparations? Was this a cross reaction of an antiserum with a common determinant shared by very different molecules? What cells actually synthesized the Ia-like molecules? Did the Ia molecules on epidermal cells indicate that they may be involved in immune surveillance? Or did this indicate that Ia molecules subserved a more general cell interaction function? I tried to approach these questions biochemically. Chapters 1 and 3 deal with the structure of Ia molecules isolated from epidermal cells. Chapter 2 deals with the origin of these molecules.

The second part of this thesis consists of studies on the rat MHC, predominantly the rat equivalent of the I region. It is informative to examine the MHC of the rat, a rodent species closely related to the mouse, yet perhaps different in some important respects. For example, some intriguing studies on wild rats suggested that the polymorphism of the rat MHC was strikingly different when compared to the mouse. In order to study the polymorphism of the rat MHC,

well-characterized antisera are essential. The production of suitable antisera is hindered by the lack of available recombinant strains of rats. I have attempted to utilize crossreactive mouse antisera in defining the rat MHC products. This approach demonstrated that the rat MHC contains two sets of class II molecules, and that the polymorphism within inbred rat strains is extensive. Further, these results indicate that mouse antisera coupled with biochemical techniques will be an important tool in analyzing the rat MHC. These results are presented in Chapters 4 and 5.

References

1. Gorer, P. A. J. Path. Bacteriol. **47:** 231 (1938).
2. Klein, J. Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag, New York (1975).
3. Klein, J. In The Major Histocompatibility System in Man and Animals, (Götze, D., ed.). Springer-Verlag, New York (1977).
4. Shreffler, D. C. Transpl. Rev. **32:** 140 (1976).
5. Coligan, J. E., Kindt, T. J., Ewenstein, B. M., Uehara, H., Nisizawa, T., and Nathenson, S. G. Proc. Nat. Acad. Sci. USA **75:** 3390 (1978).
6. Silver, J., and Hood, L. Proc. Nat. Acad. Sci. USA **73:** 599 (1976).
7. Silver, J., and Hood, L. Contemp. Top. Molec. Immunol. **5:** 35 (1976).
8. Klein, J. Science **203:** 516 (1979).
9. Zinkernagel, R. M., and Doherty, P. C. Nature **251:** 547 (1974).
10. Shearer, G. M. Eur. J. Immunol. **4:** 527 (1974).
11. David, C. S., Cullen, S. E., and Murphy, D. B. J. Immunol. **144:** 1205 (1975).
12. David, C. S., and Cullen, S. E. J. Immunol. **120:** 1659 (1978).
13. Jones, P. P., Murphy, D. B., Hewgill, D., and McDevitt, H. O. Molecular Immunol. **16:** 51 (1979).
14. Cecka, J. M., McMillan, M. M., Murphy, D. B., McDevitt, H. O., and Hood, L. Eur. J. Immunol. **9:** 955 (1979).
15. McMillan, M. M., Cecka, J. M., Murphy, D. B., McDevitt, H. O., and Hood, L. Immunogenetics **6:** 137 (1978).
16. Jones, P. P., Murphy, D. B., and McDevitt, H. O. J. Exp. Med. **148:** 925 (1978).
17. Klein, J., Geib, R., Chiang, C., and Hauptfeld, V. J. Exp. Med. **143:** 1439 (1976).
18. Möller, G. (ed.) Transpl. Rev. **30** (1976).
19. Butcher, G. W., and Howard, J. C. Nature **266:** 362 (1977).

20. Stark, O., Günther, E., Kohoutova, M., and Vojcik, L. Immunogenetics **5:** 183 (1977).

21. Blankenhorn, E. P., Cecka, J. M., Götze, D., and Hood, L. Nature **274:** 90 (1978).

22. Sporer, R., Black, G., Rigiero, C., Manson, L., and Götze, D. Immunogenetics **7:** 507 (1978).

23. Hart, D. N. J., and Fabre, J. W. Transplantation **27:** 110 (1979).

24. Günther, E., Stark, O., and Koch, C. Eur. J. Immunol. **8:** 206 (1978).

25. Blankenhorn, E. P. Thesis, Caltech (1979).

26. Blankenhorn, E. P., Cecka, J. M., Frelinger, J. G., Götze, D., and Hood, L. Eur. J. Immunol. **10:** 145 (1980).

27. Davies, H., and Butcher, G. W. Immunogenetics **6:** 171 (1978).

28. Günther, E., and Stark, O. Tissue Antigens **11:** 465 (1978).

29. Shonnard, J. W., Cramer, D. V., Poloskey, P. E., Kunz, H. W., and Gill, T. J. Immunogenetics **3:** 193 (1976).

30. Cramer, D. V., Shonnard, J. W., Davis, B. K., and Gill, T. J. Transpl. Proc. **9:** 567 (1977).

Chapter II

Epidermal Ia Molecules from the I-A and I-EC Subregions of the H-2 Complex

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Epidermal Ia Molecules from the *I-A* and *I-EC* Subregions of the Mouse *H-2* Complex

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Abstract. Immune response region-associated (Ia) antigens encoded by genes in the *I-A* or *I-EC* subregions have been detected on murine epidermal cells by indirect immunoprecipitation, using antisera produced against murine lymphoid cells. The Ia antigens encoded by genes in these subregions are composed of two polypeptides with approximate molecular weights of 33,000 and 28,000. The Ia antigens are not derived from contaminating B- or T-cell populations. The Ia molecules from lymphocytes and epidermal cells appear to have identical subunit structures and very similar, if not identical, molecular weights. The possible biological role of the Ia antigens on epidermal cells is discussed.

Introduction

The major histocompatibility or *H-2* complex of the mouse has been divided into five regions (*K*, *I*, *S*, *G*, and *D*). Genes in the *I* region are involved in regulating the immune response to antigens, graft-versus-host reactivity, T-B cell collaboration, and graft rejection (discussed in Shreffler *et al.* 1976, Klein 1975). Furthermore, *I*-region genes code for a set of serologically detected cell-surface alloantigens, designated Ia antigens. In the mouse, the *I* region has been subdivided into several subregions (*i.e.*, *I-A*, *I-B*, *I-J*, *I-E*, and *I-C*) by genetic mapping of immune response genes and by serological analysis of cell-surface antigens in recombinant strains of congenic mice (Schreffler *et al.* 1976, Murphy *et al.* 1976).

The Ia antigens isolated from mouse spleen cells by indirect immunoprecipitation and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis are composed of two subunits, the α and β polypeptides, with molecular weights of 33,000 and 28,000, respectively. Partial amino acid sequence data from the N terminus of Ia polypeptides isolated from lymphoid cells indicate that they apparently share little homology with either the K or D antigens or with immunoglobulins (Silver *et al.* 1976; McMillan *et al.* 1977).

The Ia antigens are more restricted in tissue distribution than the serologically detectable H-2K or D antigens. Whereas the K or D antigens are believed to be present on virtually every tissue, the Ia antigens have been detected only on B lymphocytes, a subpopulation of T lymphocytes, macrophages, fetal liver, epidermal cells, and spermatozoa. They are not present on red cells, platelets, muscle, brain,

and a variety of other tissues (Hämmerling *et al.* 1975, Colombani *et al.* 1976, Hauptfeld *et al.* 1974).

The function of Ia antigens has been the subject of much speculation. They have been suggested as Fc receptors (Dickler and Sachs 1974), antigen receptors (Benacerraf and McDevitt 1972), T-B cell interaction molecules (Katz *et al.* 1975), and as a component of various inhibitory or facilitating immune factors (Tada *et al.* 1976, Munro and Taussig 1975). An unanswered question that may place important constraints on the function of Ia molecules is whether the Ia molecules on different tissues are identical.

Earlier studies using antisera produced against antigens determined by genes in the entire *I* region on spleen cells have demonstrated, by direct cytotoxic tests and by adsorption analysis, that Ia antigens are present on epidermal cells (Hämmerling *et al.* 1975, Klein *et al.* 1976). Preliminary evidence indicated that some Ia-like molecules could be precipitated from a radiolabeled epidermal cell extract by indirect immunoprecipitation (Delovitch and McDevitt 1975). Our present work demonstrates that the epidermal cells express Ia antigens encoded by genes in both the *I-A* and *I-EC* subregions. Moreover, antisera directed against either of these subregions precipitate two biosynthetically labeled Ia polypeptides, the molecular weights of which are indistinguishable from those present on B lymphocytes.

Materials and Methods

Mice. B10.A(4R), B10.HTT, and B10.D2 mice were raised at the mouse colony at the University of Southern California School of Medicine. B10.A(5R) mice were the generous gift of Dr. J. Stimpfling of McLaughlin Research Institute, Great Falls, Montana. C57B1/6J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Antisera. Hyperimmune alloantisera were prepared by multiple injections of spleen, lymph node, and thymus cells as previously described (David *et al.* 1973). All alloantisera have been characterized by direct cytotoxicity and adsorption analysis, using appropriate lymphocyte targets (Frelinger *et al.* 1974). The alloantisera employed in this study and the subregions they recognize on specific targets are given in Table 1. None of the antisera utilized recognizes *I-E* or *I-C* subregion products alone, so we denote this ambiguity as *I-EC*. Rabbit anti-μ chain serum was prepared from μ chains isolated from BALB/c myeloma tumor MOPC 104E, which was the generous gift of M. Kehry, California Insitute of Technology.

Adsorption of Anti-Ia Sera by Epidermal Cells. Epidermal cell suspensions were prepared as described below. Seventy-five μl undiluted antiserum were added to a cell pellet containing 5×10^6 epidermal cells. The cells were resuspended in the serum and incubated 30 minutes on ice. The suspensions were centrifuged and the supernatant was removed. This procedure was repeated 3 times. The final supernatant was tested for residual cytotoxic activity.

Staphylococcus aureus. Cowan-I strain ATC no. 12598 (Staph-A) was prepared according to the methods described previously (Kessler 1975) as modified by Cullen and Schwartz 1976). Briefly, *Staphylococcus aureus* Cowan I (Staph A) was grown overnight in shaker flasks, washed, heat-killed, and fixed with 1.5% formaldehyde. The fixed, heat-killed Staph A was stored in aliquots at −60°C until use. The staph A is superior to rabbit anti-mouse immunoglobulin as the agent for the indirect immunoprecipitation procedure with regard to ease of handling, efficiency, and specificity (Cullen and Schwartz 1976).

Preparation and Labelling of Cells. Spleen cells were labeled as described previously (McMillan *et al.* 1977). Briefly, cells were teased through a wire screen, washed, and incubated at 2×10^7 cells/ml for 5

Table 1. The Antisera and Mouse Strains Used as Targets to Precipitate Ia Molecules

Antisera	Targets	Relevant *H-2* Regions
A.TH anti-A.TL	B10.HTT[a]	$I\text{–}E^k$, $I\text{–}C^k$
	B10.D2[b]	$I\text{–}J^d$, $I\text{–}E^d$, $I\text{–}C^d$
	A.AL	I^k
A.TL anti-A.TH	B10.HTT[c]	$I\text{–}A^s$, $I\text{–}B^s$, $I\text{–}J^s$
(A.TH × B10.HTT)F_1 anti-A.TL	B10.A(4R)	$I\text{–}A^k$
(B10 × HTI)F_1 anti-B10.A(5R)	B10.D2	$I\text{–}E^d$, $I\text{–}C^d$
(A × B10.D2)F_1 anti-B10.A(5R)	C57B1/6J	K^b, $I\text{–}A^b$, $I\text{–}B^b$

[a] Since Ia-like membrane antigens have not been demonstrated for the *S* or *G* regions in lymphocytes, this antiserum is probably reacting principally with *I*-region molecules

[b] This antiserum potentially has reactivity with Ia.15 on B10.D2 cells. However, absorption with B10.HTT cells (Ia.7) leaves no residual activity on B10.D2 cells. Thus, this sera reacts primarily with Ia.7 and the molecule(s) precipitated is probably an *I–C* subregion product. Although crossreactions of $I\text{–}J^k$ and $I\text{–}J^d$ have been reported (Frelinger *et al.* 1976), *I–J* is expressed on only a small subpopulation of lymphocytes and probably does not contribute to the observed reactions

[c] Since no Ia specificities can be attributed to the *I–B* subregion and little compelling evidence can be produced for its existence, we have designated all reactions with *I–A* and *I–B*, *I–A*

hours in Hanks' balanced salt solution supplemented with 10 mM Hepes, Eagle's minimal essential media amino acids (minus tyrosine and leucine), 5% dialyzed calf serum, and 1 mCi each of ^{3}H-tyrosine and ^{3}H-leucine. T cells were isolated as described previously (Julius *et al.* 1973) and labeled as described above for spleen cells. Epidermal cells were prepared, using published procedures (Scheid *et al.* 1972), from mouse tails. Every cell preparation was examined microscopically, and each showed a morphology typical of epidermal cells. Cells were typically 2 or 3 lymphocyte diameters with prominent cytoplasm, and thus are easily distinguished from lymphocytes. Preparations were free of lymphocyte contamination. No small round cells were seen in several hundred epidermal cells observed. Only occasional red cells were seen, indicating little or no contamination of the epidermal cells preparations by peripheral blood cells. Mice with tail scars or lesions were excluded as cell donors. Epidermal cells were labeled as described above for spleen cells, but at a concentration of 1×10^7 cells/ml. Spleen or T-cell preparations were lysed in a volume equal to the incubation mixture, with 0.01 M Tris, 0.15 M NaCl, and 0.5% NP-40 at pH 7.4. Epidermal cells were lysed with the same buffer but at a concentration of 3×10^6 cells/ml lysis buffer. The debris was removed by centrifugation.

Lens culinaris Affinity Chromatography. Lens culinaris lectin was isolated from the common lentil (Hayman and Crumpton 1972) and coupled to CNBr-activated Sepharose 4B (Cuatrecasas *et al.* 1968) at 1.5 mg lectin/ml of settled Sepharose 4B. Nonidet (Shell trademark) P40 (NP-40) extracts were passed over a 10-ml lectin column at 4°C, and the bound fraction was eluted with 0.1 M alpha-methyl-D-mannoside. This fraction was concentrated to the original volume in a B15 Minicon filter (Amicon). This concentrated material was used in subsequent precipitations.

Immunoprecipitations. The lectin-purified extract was incubated with antisera, typically 400 μl NP-40 extract and 20 μl of a specific alloantisera, for 2 hours, and subsequently precipitated with Staph A for 1 hour, usually 200 μl 10% Staph A solution. The Staph A was washed 3 times and the bound material eluted with a mixture of 2% SDS, 2% 2-mercaptoethanol, and 50 mM Tris pH 6.8. This sample was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). The gels were sliced into 1-mm sections, and the radioactivity was eluted and then counted in a Beckman LS230 scintillation counter.

Results

Ia Molecules Encoded by Genes in Both the I-A and I-EC Subregions Can Be Isolated from Epidermal Cells

Figure 1 shows the SDS-polyacrylamide gel analysis of labeled immunoprecipitates, employing a variety of antisera with restricted specificities (Table 1). Ia molecules encoded by genes in the $I\text{-}A^s$ (Fig. 1a), $I\text{-}A^k$ (Fig. 1b), and $I\text{-}A^b$ (Fig. 1c) subregions were isolated and characterized on SDS-polyacrylamide gels. Ia molecules from the $I\text{-}EC^k$ (Fig. 1d) and $I\text{-}EC^d$ (Fig. 1e) subregions were characterized in a similar manner. The sera and strains utilized as targets are given in the legend to Figure 1. The $H\text{-}2^{ls}$ and $H\text{-}2^d$ haplotypes share the Ia.7 specificity, and this is the cause of the reaction of (B10 × HTI)F_1 anti-B10.A(5R) with the B10.D2 extract (David *et al.* 1975; Fig. 1e). The peaks from the B10.D2 extract are substantially larger because a larger amount of cell extract was utilized for that precipitation relative to the other precipitation seen in Figure 1.

The Ia molecules encoded by genes in the *I-A* or *I-EC* subregions isolated from lymphocytes or epidermal cells are similar, if not identical, in their electrophoretic

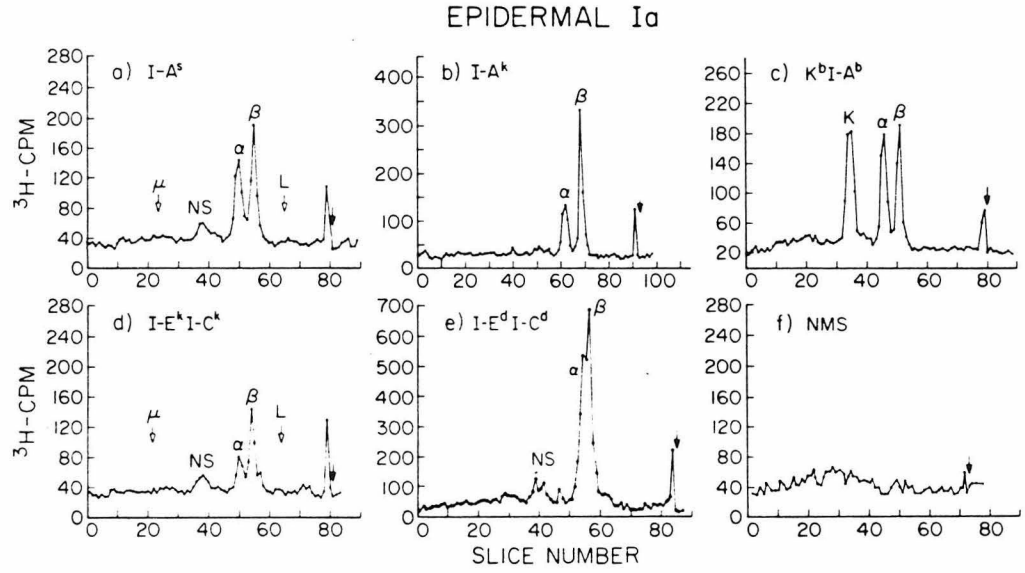

Fig. 1a–f. SDS-polyacrylamide gel electrophoresis patterns of Ia molecules isolated from epidermal cells by immunoprecipitation. Restricted Ia antisera and Staph A were used to precipitate Ia molecules from lectin-purified epidermal cell extracts. **a)** $I\text{-}A^s$ molecules detected by A.TL anti-A.TH on a B10.HTT extract representing 1.3 × 10^6 cells. **b)** $I\text{-}A^k$ molecules detected by (A.TH × B10.HTT)F_1 anti-A.TL on a B10.A(4R) extract representing 3 × 10^6 cells. **c)** K^b $I\text{-}A^b$ molecules detected by (A × B10.D2)F_1 anti-B10.A(5R) on a B10.A(5R) extract representing 1.3 × 10^6 cells. **d)** $I\text{-}E^k$ $I\text{-}C^k$ molecules detected by A.TH anti-A.TL on a B10.HTT extract representing 1.3 × 10^6 cells. **e)** $I\text{-}E^d$ $I\text{-}C^d$ molecules detected by (B10 × HTI)F_1 anti-B10.A(5R) on a B10.D2 extract representing 7.5 × 10^6 cells. **f)** Control employing normal mouse serum on a B10.A(5R) extract representing 1.3 × 10^6 cells. (⇨) Arrows indicate MOPC 104E heavy-chain (μ) and light-chain markers. (➡) Arrows indicate the dye front. NS represents the nonspecific peaks. All the extracts were precipitated by Staph A with the exception of the B10.D2 extract, which was preprecipitated with rabbit anti-μ chain antiserum and Staph A

behavior. Two molecular-weight components were resolved by this gel procedure in every case examined, though in general, the *I-EC* subregion components migrate with more similar Rf values than the components encoded in the *I-A* subregion. This is precisely the pattern seen in the Ia molecules isolated from spleen cells (Fig. 2). The molecular weights of splenic and epidermal Ia on SDS-polyacrylamide gels are identical within experimental error (±1000).

Epidermal Cells Remove Activity of Anti-Ia Serum

A.TH anti-A.TL (anti-I^k) serum was adsorbed with B10.S and A.TL epidermal cells. Adsorption of this serum with A.TL epidermal cells reduced the cytotoxic titer

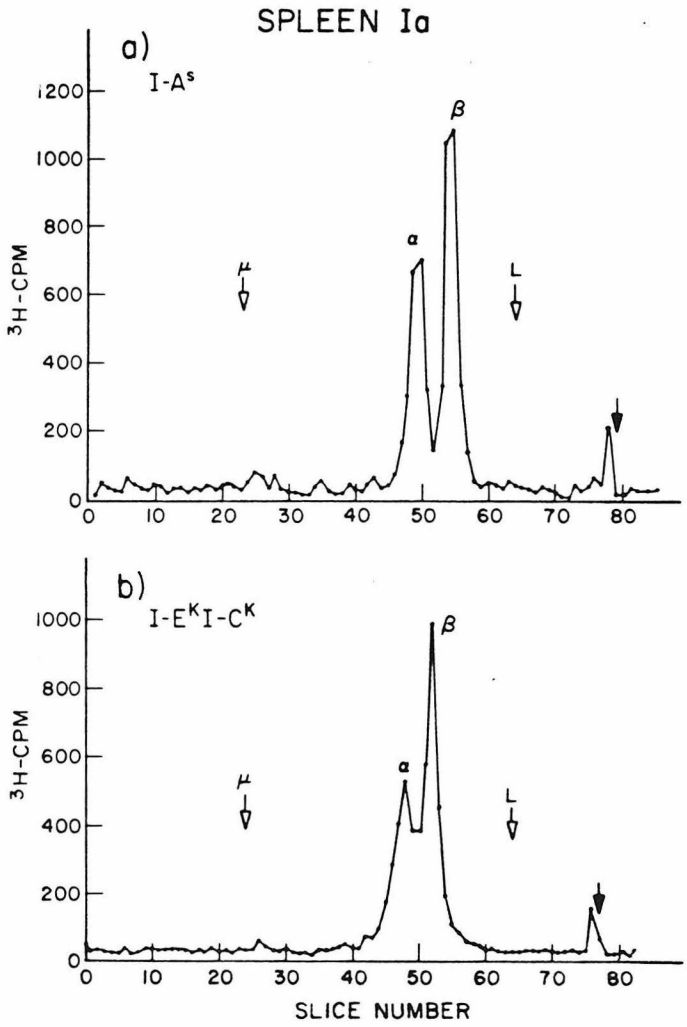

Fig. 2a and b. SDS-polyacrylamide gel electrophoresis patterns of Ia molecules from spleen. Lectin-purified B10.HTT spleen cell extracts representing 1 × 10^6 cells were precipitated with **a**) A.TL anti-A.TH, detecting *I-A^s* molecules, and **b**) A.TH anti-A.TL, detecting *I-E^k I-C^k* molecules. See the legend to Figure 1 for a description of the various markers

on lymph node lymphocytes from greater than 1/640 to less than 1/20. Adsorption with B10.S epidermal cells left a residual titer of greater than 1/320. Thus, A.TL epidermal cells share all the serological specificities present on A.TL lymph nodes cells detected by cytotoxicity.

Transplantation Antigens Can Be Isolated from Epidermal Cells

The peak of molecular weight 45,000 in Figure 1C is expected, because the antiserum used reacts with the K^b molecules. Thus, we have also shown that the serologically detectable K transplantation antigen is present on epidermal cells. The irregular peak of 45,000 molecular weight seen in some of the other extracts could be decreased in size by preclearing with Staph A, but was never completely eliminated. This peak probably represents nonspecific precipitation of actin (P. Jones, personal communication). We can eliminate this nonspecific peak by using the same procedure on spleen cell extracts (Fig. 2).

Epidermal Ia Molecules Are Not Contributed by B Cells (Ig-Positive Cells)

B10.D2 epidermal cells from mouse tails were isolated in the standard manner. This preparation was divided into two equal aliquots, one of which was labeled as described in *Materials and Methods*. C57B1/6J (B6) spleen lymphocytes were added

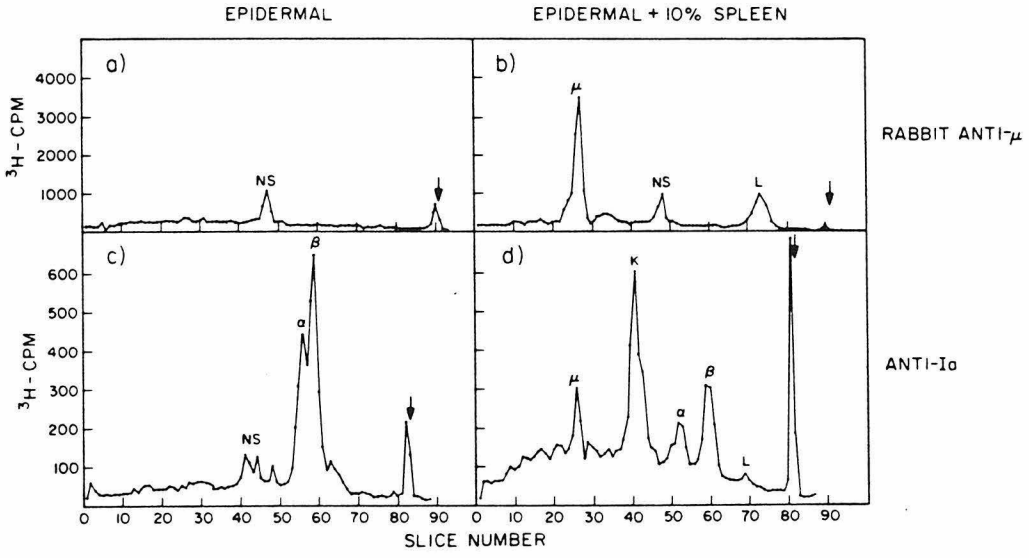

Fig. 3a–d. Mixture experiments with B cells and epidermal cells. **a**) SDS-polyacrylamide gel electrophoresis patterns of a lectin-purified B10.D2 epidermal cell extract representing 1.5×10^7 cells precipitated with rabbit anti-μ serum. **b**) Lectin-purified extract representing 1.5×10^7 B10.D2 epidermal cells and 1.5×10^6 B6 spleen cells, precipitated with rabbit anti-μ serum. **c**) One-half of the supernatant after the precipitation in (**a**) representing 7.5×10^6 B10.D2 epidermal cells precipitated with A.TH anti-A.TL serum. **d**) One-half of the supernatant after the precipitation in (**b**) representing 7.5×10^6 B10.D2 epidermal cells and 7.5×10^5 B6 spleen cells precipitated with (A × B10.D2)F_1 anti-B10.A(5R) serum. NS represents the nonspecific peaks. (➡) Arrows indicate the dye front

to the second aliquot to a concentration of ten percent of the epidermal cell number, and then labeled as the first aliquot. Both preparations were precipitated with rabbit anti-μ heavy chain. The analyses of these precipitations are shown in Figures 3a and 3b. One-half of the supernatant aliquot of B10.D2 tail cells was then precipitated with A.TH anti-A.TL (Fig. 3c). One-half of the supernatant from the aliquot of B10.D2 tails contaminated with B6 spleen cells was precipitated with (A × B10.D2)F_1 anti-B10.A(5R) (Fig. 3d). This serum recognizes only the Ia antigen from the spleen cells, since the antisera was made in an (A × B10.D2)F_1 mouse, and thus, is genetically blocked for reactivity with the B10.D2 Ia components present on the epidermal cells. The analysis of precipitates by SDS-polyacrylamide gel electrophoresis is shown in Figures 3c and 3d. No heavy-chain or light-chain peak is seen from twice the amount of extract necessary to observe an Ia peak from the B10.D2 tails alone, as can be seen by comparing Figures 3a and 3c. Furthermore, the heavy-chain peak seen in the artificially contaminated tail cells preparation is substantially larger (~fourfold) than the Ia peaks contributed by the spleen cells (Figs. 3b and 3d). If the Ia molecules in Figure 3c are from Ig-positive cells, we should be able to see easily a heavy-chain peak in Figure 3a, but this is not observed. Thus, contamination from B cells (Ig-positive) is not the source of the Ia antigen detected from the epidermal cell preparations. This finding is consistent with the absence of any fluorescent cells (0/40) when the epidermal cells were treated with fluorescein-labeled rabbit anti-mouse Ig (J.A. Frelinger, unpublished data).

T Cells Are Probably Not the Source of the Ia Molecules Seen in These Experiments

T cells were enriched by a passage of spleen cells through a nylon wool column (Julius *et al.* 1973). The labeled extract of the partially purified T cells was precipitated with both rabbit anti-μ chain and A.TH anti-A.TL. The results are shown in Figures 4a and 4b, respectively. The Ia present in Figure 4b could be explained by the presence of contaminating B cells, as evidenced by the amount of heavy chain and light chain in Figure 4a. The ratio of immunoglobulin to Ia molecules in the T-cell preparation is the same as that found in B or spleen cell preparations. This does

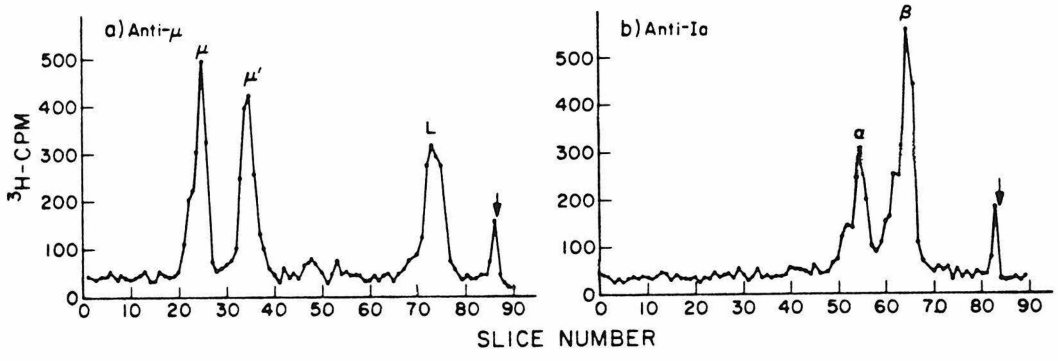

Fig. 4a and b. SDS-polyacrylamide gel electrophoresis patterns of immunoprecipitations from lectin-purified A.AL nylon wool T cell extracts representing 2 × 10^6 cells. **a)** T cells precipitated with rabbit anti-104E serum. **b)** T-cell extract precipitated with A.TH anti-A.TL serum. The μ' peak in Figure 4a probably represents a degradation product of the μ chain. (➡) Arrows indicate the dye front

not suggest that T cells lack Ia molecules. Others have estimated, using analogous techniques, that thymocytes have from 50 to 100 times less Ia per cell than B cells (Schwartz *et al.* 1977). However, within experimental limits, the amount of Ia precipitated in Figure 4b could be explained by the ten percent contaminating B cell estimated by direct immunofluorescence in this T-cell preparation. No Ia molecules could be ascribed to T cells in a cell preparation specifically enriched for T cells. We can rule out contamination of the epidermal preparations by more than five percent lymphocytes by morphological criteria. Thus, T cells are not a likely source of the Ia antigens seen in these epidermal cell extracts.

Discussion

Epidermal Ia Molecules Coded by Genes in the I-A and I-EC Subregions are Very Similar to Their Lymphoid Counterparts

Two Ia polypeptides can be isolated from the *I-A* and *I-EC* subregions from epidermal and spleen cell suspensions, using antisera prepared against lymphocytes. Thus, the epidermal and lymphoid Ia molecules share serological determinants. Further, the Ia molecules from epidermal and spleen cells share the charactertistic two-chain polypeptide pattern on SDS-polyacrylamide gels. The molecular weights of the Ia polypeptides isolated from spleen and epidermal cells are apparently identical. In addition, the size and shape of the subunit peaks from the two cell types are similar, when labeled with ^{3}H-tyrosine and ^{3}H-leucine, suggestive, although not definite proof, that the polypeptides have similar amino acid compositions. Finally, the Ia molecules from the *I-EC* subregion consistently migrate closer together than the molecules from the *I-A* subregion from both spleen and epidermal cells (Figs. 1 and 2).

Which Cells in the Epidermal Cell Preparation Are Responsible for the Synthesis of the Ia Molecules Seen in Our Experiments?

Tail cells were isolated by a procedure which characterized these cells as epidermal by their morphology in the electron microscope (Scheid *et al.* 1972). The Ia antigens are not derived from T or B cells (Figs. 3 and 4). However, our experiments give no direct information about either the proportion or the type of cells in the epidermis that are Ia-positive. Two previous reports of the expression of *I*-region markers on murine epidermal cells suggest that significant amounts of Ia are present on most epidermal cells. These studies employed direct cytotoxic tests to demonstrate that more than 80 percent of mouse epidermal cells have cell-surface Ia antigens which lead to cell lysis by anti-Ia sera and complement (Hämmerling *et al.* 1975, Klein *et al.* 1976). Indirect immunofluorescence studies suggest that more than 90 percent of cells in the epidermal cell suspensions are Ia-positive (J.A. Frelinger, unpublished data). Quantitative adsorptions suggest that epidermal cells have only two to four times fewer Ia molecules per cell than B cells (Klein *et al.* 1976). Unless one postulates cells with significantly greater amounts of Ia antigens than B cells, this adsorption data is not consistent with a small population of Ia-positive cells in the epidermis. Thus, the evidence in mice strongly suggests that most of the cells in the epidermis are Ia-positive.

The percentage of Ia-positive cells in human epidermis may be much lower than in mouse epidermis. Recent studies investigated which cells, keratinocytes, melanocytes, or Langerhans cells in the human epidermis are Ia-positive. Indirect immunofluorescence indicates only two to three percent of the cells in the epidermis, the macrophage-like Langerhans cells, are HLA-D-positive (Rowden and Sullivan 1977, Klaresky *et al.* 1977). The HLA-D region appears to encode the human equivalent of the Ia antigens. The reason for the apparent discrepancy in the frequency of Ia-positive cells in the epidermis of mouse and human is not clear. It might represent a species-associated difference in the distribution of Ia antigens. Alternatively, we believe that it probably reflects technical differences in the experimental system, such as differences in the titer of the antisera employed and the sensitivity of the detection system.

The Apparent Identity of Ia on Epidermal and B Cells Has Interesting Implications for the Function of Ia Molecules

There is no evidence which suggests any function for Ia molecules on epidermal cells, either immunological or other. Fluorescence studies in the human indicate that epidermal Langerhans cells are Ia-positive (Rowden and Sullivan 1977, Klaresky *et al.* 1977) and Fc-positive (Stingl *et al.* 1977, Rowden and Sullivan 1977). Other studies on guinea pigs show that Langerhans cells have an affinity for certain antigenic metals and amines (Shelly and Juhlin 1976). Furthermore, there is an increased number of Langerhans cells in the draining lymph nodes relative to controls in passively sensitized guinea pigs when they are rechallenged with antigen (Silberberg-Sinakin *et al.* 1976). Thus, investigators have suggested that Langerhans cells may function in a fashion analogous to macrophages and be involved in the uptake and transport of antigenic material from the epidermis to the lymphatic system. However, Langerhans cells probably represent only a subset of the Ia-positive cells in the mouse epidermis, which raises the question as to the function of the remaining Ia-positive cells. An interesting speculation is that the Ia molecules on skin cells are present to facilitate the stimulation of T cells associated with cells present in the epidermis. This hypothesis is consistent with the finding that the transfer of delayed-type hypersensitivity in mice requires *I-A* region compatibility of responding T cells and sensitizing macrophages (Miller *et al.* 1976). Besides predicting that the Ia molecules on epidermal and B cells would be identical, this also suggests that other tissues continuously exposed to environmental immunogens, such as the epithelial surfaces of the gut and lungs, should be Ia-positive. An alternative hypothesis is that the Ia molecules on the epidermis may assist in an immune surveillance function of T cells, possibly by stabilizing interactions between T and epidermal cells. In any case, theories as to the function of Ia molecules must take into account the presence of epidermal Ia molecules that appear to be similar if not identical to their counterparts on lymphoid cells.

We have demonstrated by immunoprecipitation techniques the presence on epidermal cells of Ia molecules similar to those found on B cells from both the *I-A* and *I-EC* subregions. These immunoprecipitation data are consistent with the serological and skin graft rejection data which indicate that Ia molecules are present on epidermal cells (Hämmerling *et al.* 1975, Klein *et al.* 1976). This system will permit the first detailed analysis of nonlymphoid Ia molecules. We are in the process of peptide map analyses and two-dimensional gel electrophoresis studies to determine if the epidermal and lymphoid Ia are, in fact, identical.

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References

Benacerraf, B. and McDevitt, H.O.: Histocompatibility-linked immune response genes. *Science 174*:273–279, 1972.

Colombani, J., Colombani, M., Shreffler, D.C., and David, C.: Separation of anti-Ia (I-region associated antigens) from anti-H-2 antibodies in complex sera by absorption on blood platelets. Description of three new Ia specificities. *Tissue Antigens 7*:74–85, 1976

Cuatrecasas, P., Wilchek, H., and Anfinsen, C.B.: Selective enzyme purification by affinity chromatography. *Proc. Natl. Acad. Sci. U.S.A. 61*:636–643, 1968

Cullen, S.E. and Schwartz, B.D.: An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing Staphylococci. *J. Immunol. 117*:136–142, 1976

David, C.S., Shreffler, D.C., and Frelinger, J.A.: New lymphocyte antigen system (LNA) controlled by the *Ir* region of the mouse *H-2* complex. *Proc. Natl. Acad. Sci. U.S.A. 70*:2509–2514, 1973

David, C.S., Cullen, S.E., and Murphy, D.B.: Serologic and biochemical studies of the Ia system of the mouse *H-2* gene complex. Further evidence for an *I-C* subregion. *J. Immunol. 114*:1205–1209, 1975

Delovitch, T. and McDevitt, H.O.: Isolation and characterization of murine Ia antigens. *Imunogenetics 2*:39–52, 1975

Dickler, H.B. and Sachs, D.H.: Evidence for identity or close association of the Fc receptor of B lymphocytes and alloantigens determined by the Ir region of the *H-2* complex. *J. Exp. Med. 140*:779–796, 1974

Frelinger, J.A., Niederhuber, J.E., David, C.S., and Shreffler, D.C.: Evidence for the expression of Ia (H-21 associated) antigens on thymus-derived lymphocytes. *J. Exp. Med. 140*:1273–1284, 1974

Frelinger, J.A., Niederhuber, J.E., and Shreffler, D.C.: Effects of anti-Ia sera on mitogenic responses. III. Mapping the genes controlling the expression of Ia determinants on Con A reactive cells to the I-J subregion. *J. Exp. Med. 144*:1141–1146, 1976

Hämmerling, G.J., Mauve, G., Goldberg, E., and McDevitt, H.O.: Tissue distribution of Ia antigens: Ia on spermatozoa, macrophages, and epidermal cells. *Immunogenetics 1*:428–437, 1975

Hauptfeld, V., Hauptfeld, M., and Klein, J.: Tissue distribution of *I* region associated antigens in the mouse. *J. Immunol. 113*:181–188, 1974

Hayman, M.J. and Crumpton, M.J.: Isolation of glycoproteins from pig lymphocyte plasma membrane using *Lens culinaris* phytohemagglutinin. *Biochem. Biophys. Res. Commun. 47*:923–930, 1972

Julius, M.H., Simpson, E., and Herzenberg, L.A.: A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol. 3*:645–649, 1973

Katz, D.H., Graves, M., Dorf, M.E., Dimuzio, H., and Benacerraf, B.: Cell interactions between histo-incompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J. Exp. Med. 141*:263–268, 1975

Kessler, S.: Protein A-antibody adsorbent for isolation of cellular antigens. *J. Immunol. 115*:1617–1624, 1975

Klaresky, L., Tjerlund, U.M., Forsum, U., and Peterson, P.A.: Epidermal Langerhans cells express Ia antigens. *Nature 268*:248–250, 1977

Klein, J.: *Biology of the Mouse Histocompatibility-2 Complex*. Springer-Verlag, New York, 1975

Klein, J., Geib, R., Chiang, C., and Hauptfeld, V.: Histocompatibility antigens controlled by the *I* region of the murine *H-2* complex. I. Mapping of the *H-2A* and *H-2C* loci. *J. Exp. Med. 143*:1439–1452, 1976

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*:680–685, 1970

McMillan, M., Cecka, J.M., Murphy, D.B., McDevitt, H.O., and Hood, L., Structure of murine Ia antigens: partial N-terminal amino acid sequence of products of the I-E/I-C subregion. *Proc. Natl. Acad. Sci. U.S.A.*, in press, 1978

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., and Gamble, J.: Role of major histocompatibility complex gene products in delayed type hypersensitivity. *Proc. Natl. Acad. Sci. U.S.A. 73*:2486–2490, 1976

Munro, A.J. and Taussig, M.J.: Two genes in the major histocompatibility complex control immune response. *Nature 256*:103–106, 1975

Murphy, D.B., Herzenberg, L.A., Okumura, K., Herzenberg, L.A., and McDevitt, H.O.: A new *I* subregion (*I-J*) marked by a locus (*Ia-4*) controlling surface determinants on suppressor T lymphocytes. *J. Exp. Med. 144*:699–712, 1976

Rowden, G., Lewis, M.G., and Sullivan, A.K.: Ia antigen expression on human epidermal Langerhans cells. *Nature 268*:247–248, 1977

Scheid, M., Boyse, E.A., Carswell, E.A., and Old, L.J.: Serologically demonstratable alloantigens of mouse epidermal cells. *J. Exp. Med. 135*:938–955, 1972

Schwartz, B.D., Kask, A.M., Sharrow, S.O., David, C.S., and Schwartz, R.H.: Partial chemical characterization of Ia antigens derived from murine thymocytes. *Proc. Natl. Acad. Sci. U.S.A. 74*:1195–1199, 1977

Shelly, W.B. and Juhlin, L.: Langerhans cells form a reticuloepithelial trap for external contact antigens. *Nature 261*:46–47, 1976

Shreffler, D.C., David, C.S., Cullen, S.E., Frelinger, J.A., and Niederhuber, J.E.: Serological and functional evidence for further subdivision of the *I* regions of the *H-2* gene complex. *XLI Cold Spring Harbor Symposium on Quantitative Biology 41*:477–487, 1976

Silberberg-Sinakin, I., Thorbecke, G.J., Baer, R.L., Rosenthal, S.A., and Berezowsky, V.: Antigen-bearing Langerhans cells in skin, dermal lymphatics and in lymph nodes. *Cell. Immunol. 25*:137–151, 1976

Silver, J., Cecka, J.M., McMillan, M., and Hood, L.: Chemical characterization of products of the *H-2* complex. *XLI Cold Spring Harbor Symposium on Quantitative Biology 41*:369–377, 1976

Stingl, G., Wolff-Schreiner, E.C., Pichler, W.J., Gescnait, F., Knapp, W., and Wolff, K.; Epidermal Langerhans cells bear Fc and C3 receptors. *Nature 268*:245–246, 1977

Tada, T., Taniguchi, M., and David, C.S.: Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. IV. Special subregion assignment of the gene(s) that code for the suppressive T-cell factor in the *H-2* histocompatibility complex. *J. Exp. Med. 144*:713–725, 1976

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Chapter III

Mouse Epidermal Ia Molecules Have a Bone Marrow Origin

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Mouse epidermal Ia molecules have a bone marrow origin

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The major histocompatibility or H-2 complex of the mouse is divided into five regions (K, I, S, G and D)[1]. Genes in the I region regulate immune responses[2]. The I region has several subregions which are designated I-A, I-B, I-J, I-E and I-C. The I-A and I-E subregions also code for a set of serologically detected cell-surface alloantigens, designated Ia antigens[3]. The relationship between the genes regulating the immune response and those encoding the serologically detectable alloantigens is still unknown. A number of species including man, rats and guinea pigs contain genetic regions apparently equivalent to the murine I region. Ia molecules are integral cell-surface glycoproteins that consist of two subunits of approximate molecular weights 35,000 (α) and 28,000 (β). Unlike the classical transplantation antigens which are present on almost all cells, the Ia antigens are found primarily on cells of the immune system—lymphocytes and macrophages[4-6]. A notable exception has been the demonstration of Ia antigens in mice, or Ia-like antigens in other mammals, on epidermal cells[6-13]. There is controversy about the numbers of Ia-positive cells in the epidermis. Fluorescence studies in humans[11,12], guinea pigs[14] and mice[15] indicate that only about 5% of epidermal cells are Ia positive. These cells were identified by morphological criteria as the macrophage-like Langerhans cells. However, cytotoxicity studies in mice using anti-Ia sera indicate that a majority of epidermal cells (up to 90%) are Ia positive[6-8]. The reason for this discrepancy is not known. Here we demonstrate that the epidermal Ia molecules are synthesised by bone marrow-derived cells, presumably Langerhans cells.

We replaced the entire population of bone marrow-derived cells in one animal with bone marrow cells from a second animal capable of synthesising Ia molecules distinct from the first. These chimaeric animals are constructed by the injection of donor bone marrow cells into lethally irradiated recipients. In chimaeric animals all cells except those of bone marrow origin are of the recipient genotype. We reasoned that if the Ia molecules synthesised in epidermal cell preparations (mouse tail cells) are synthesised by bone marrow-derived cells, the Ia molecules isolated from long-term (>3 months) chimaeras should be of donor origin. Alternatively, if the Ia molecules are synthesised by a majority of epidermal cells, the Ia molecules should be of recipient origin.

Our experimental design requires almost complete replacement of recipient bone marrow-derived cells with donor cells. Direct tests confirmed that this had occurred (Table 1). Two types of chimaeric animal were constructed: parental (P) cells were transplanted to F_1 offspring ($P \rightarrow F_1$) and vice versa ($F_1 \rightarrow P$). For the $P \rightarrow F_1$ chimaeras, the analysis of bone marrow-derived cells used cytotoxicity and haemagglutination assays. In the case of the $F_1 \rightarrow P$ chimaeras, the donor cells in the recipient were measured by haemagglutination. In both cases, the results indicated that the haematopoietic system was almost completely reconstituted with donor cells (Table 1).

We isolated epidermal Ia molecules by indirect immunoprecipitation using mouse alloantisera as previously described[10]. The specificity of the antisera is critical in determining whether the Ia is contributed by donor or recipient cells in these chimaeras. The sera were characterised extensively by cytotoxicity,

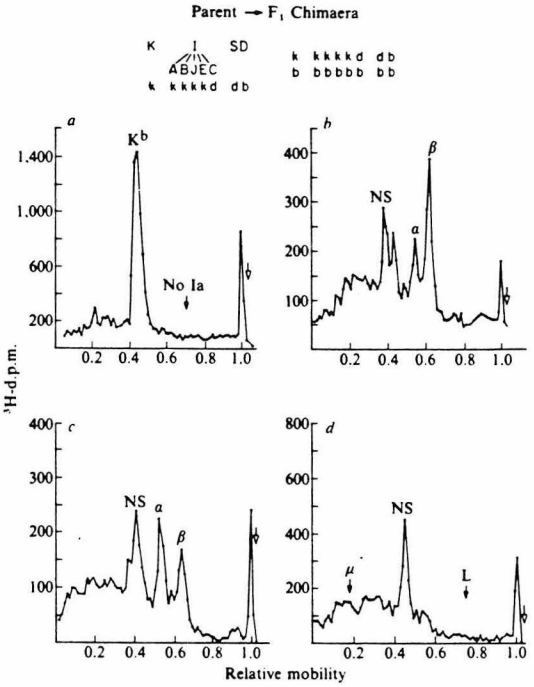

Fig. 1 SDS-polyacrylamide gel electrophoresis of epidermal Ia molecules isolated from parent → F_1 bone marrow chimaeras. Epidermal Ia was isolated as previously described[10]. Briefly, single cell suspensions of trypsinised epidermal cells were biosynthetically labelled with ^{3}H-amino acids and lysed in 0.5% NP40. The glycoprotein fraction was isolated by *Lens culinaris* lectin affinity chromatography, concentrated fivefold in a B-15 Minicon, and indirectly immunoprecipitated with specific antisera and fixed *Staphylococcus aureus* (Cowan I strain). The parent → F_1 chimaera extracts were precipitated with $(A \times B10.D2)F_1$ α B10.A(5R) [αK^bI-A^bI-B^b] (*a*) $(B10 \times HTI)F_1$ α B10.A(5R) [αI-E^d I-C^k] (*b*) $(A.TH \times B10.HTT)F_1$ α A.TL[αI-A^kI-B^kI-J^k] (*c*) rabbit anti-MOPC 104E [R$\alpha\mu$] (*d*). ↓Arrows indicate MOPC 104E heavy chain (μ) and light chain markers; | arrows indicate the dye front. NS represents nonspecific 45K peaks, possibly actin.

absorption and immunoprecipitation (data not shown). They were tested on strains identical to those used in these experiments to demonstrate unequivocally that the observed reaction was specific for recipient or donor strains. An F_1 animal [B10.A(1R) × B10] is heterozygous for the H-2 complex and expresses Ia molecules encoded by both the $H\text{-}2^{h1}$ and $H\text{-}2^b$ haplotypes (Fig. 1). In F_1 chimaeras ($P \rightarrow F_1$), only Ia molecules from the donor animal can be isolated from epidermal cells. Both I-A and I-E subregion products can be detected (Fig. 1*b*, *c*). In these chimaeras, we cannot detect Ia molecules of the $H\text{-}2^b$ haplotype from the B10 parent (Fig. 1*a*). In contrast, transplantation antigens of both haplotypes can readily be detected in these chimaeric animals. The results in Fig. 1*a* also provide direct evidence that transplantation molecules (K^b) can be synthesised by a non-bone marrow-derived cell. We should stress that immunoprecipitation with a rabbit anti-μ serum showed no evidence of contaminating IgM-positive cells in the epidermal cell population (Fig. 1*d*). Thus, the donor Ia must be derived from a population of bone marrow cells distinct from B cells.

In the chimaera described above, we could only demonstrate a loss of one haplotype of Ia antigens from tail cell preparations derived from $P \rightarrow F_1$ chimaeras. To demonstrate directly the

appearance of new Ia antigens not present in the host, $F_1 \rightarrow P$ chimaeras were constructed. The $[B6 \times A]F_1$ animal has Ia molecules of the H-2^b (B6) and H-2^a (A) haplotypes. In $[B6 \times A]F_1 \rightarrow$ B6 chimaeras, we could detect the presence of Ia molecules of both the H-2^a and H-2^b haplotypes by immunoprecipitation. I-A Subregion products from both H-2^b and H-2^a haplotypes were easily detected (Fig. 2*a*, *b*). The A^k molecules can only be derived from the bone marrow cells. Both A^k and E^k molecules are present in these chimaeras (E^k not shown). Thus, new epidermal Ia antigens are derived from the donor bone marrow cells. These data from the $P \rightarrow F_1$ and $F_1 \rightarrow P$ chimaeras strongly suggest that the Ia molecules from chimaeric epidermal cells originate from bone marrow-derived cells.

The original reports of Ia molecules on mouse epidermal cells were based on adsorption analysis and antibody-mediated cytotoxicity using dye exclusion[6]. Other investigators independently confirmed these results[7,8]. Using an indirect immunofluorescence assay, investigators working with guinea pigs, man and mouse, have suggested that the Ia is confined to a small subpopulation of epidermal cells, the macrophage-like Langerhans cells[11,12,14,15]. In guinea pigs, biosynthetically labelled Ia molecules can only be immunoprecipitated from a Langerhans cell-enriched fraction, and not from a Langerhans cell-depleted fraction of epidermal cells[16]. Our experiments suggest that the biosynthetically labelled Ia molecules are derived from a cell of bone marrow origin. These observations suggest that a small number of cells in the epidermis are derived from the bone marrow and express Ia molecules. Although we have no direct evidence for the bone marrow origin of Langerhans cells, the fluorescence studies in guinea pigs, man and mouse[15], together with the present results, suggest that the Langerhans cell produces the epidermal Ia molecules and has a bone marrow origin. This possibility is not surprising considering the macrophage-like functions ascribed to the Langerhans cells[17,18]. Macrophages are generally thought to be derived from bone marrow precursors. However, our experiments detect only newly synthesised Ia molecules. The sensitivity of detection is a function of both the number of Ia molecules and their turnover rate. We are aware that Ia molecules could be synthesised by non-bone marrow-derived cells, and not detected in our experiments, if the Ia molecules were in much lower concentration per cell or turning over more slowly.

Table 1 Source of haematopoietic cells in chimaeric mice

Target	Anti-K^b H-2.33*	Anti-D^b H-2.2†	Anti-K^k H-2.23*	Anti-D^d H-2.4†
Donor→Recipient				
IR→(B10×1R)F_1	<10 (<10)	NT	320 (>90)	NT
(B6×A)F_1 → B6	NT	80	NT	>320
Controls				
1R	<10 (<10)	160	320 (>90)	<10
B10	>640 (>80)	160	<10 (<10)	<10
A	<10 (<10)	<10	320 (>90)	>320
(B6×A)F_1	NT	80	NT	>320
(B10×1R)F_1	>640 (>90)	NT	320 (>90)	NT

Chimaeras were produced as described by Von Boehmer[20]. Mice were irradiated with 925 rads from a linear accelerator and reconstituted with $1-2 \times 10^7$ anti-mouse brain ($\alpha\theta$) plus complement-treated bone marrow cells. Mice were tested for chimaerism after 3 months. NT, not tested.

*Tested by dye exclusion microcytotoxicity on peripheral blood leukocytes. Numbers represent reciprocal of the antiserum dilution giving 50% killing of targets and in parentheses, the maximum attainable killing. Sera for cytotoxicity: anti-H-2.33 (A×B10.D2)F_1 anti-B10.A(5R); anti-H-2.23, (B10×A.TL)F_1 anti-A.AL.

†Tested by polyvinylpyrrolidone (PVP) haemagglutination of red blood cells. Number represents the reciprocal of the greatest antiserum dilution which permits detectable agglutination reaction. Sera for haemagglutination: anti-H-2.2, (A×B10.D2)F_1 anti-B10.A(2R); anti-H-2.4, (B10.A(1R)×A.SW)F_1 anti-B10.A.

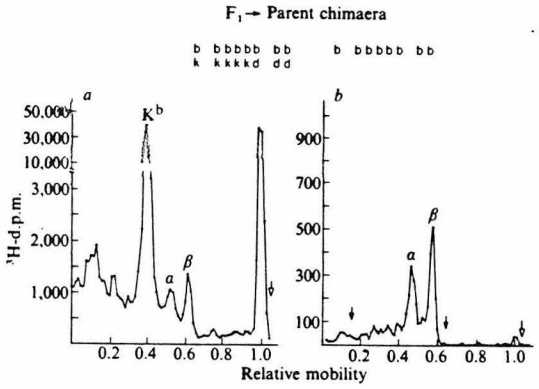

Fig. 2 SDS-polyacrylamide gel electrophoresis of epidermal Ia molecules isolated from $F_1 \rightarrow$ parent bone marrow chimaeras. The $F_1 \rightarrow$ parent chimaera extracts were precipitated with (A×B10.D2)F_1 αB10.A(5R) [$\alpha K^b I\text{-}A^b I\text{-}B^b$] (*a*) and CWBαC3H hybridoma 10–2.15 [$\alpha\text{-}A^k$] (*b*). This reagent has been characterised to react with A^k (ref. 21). We tested the hybridoma reagent using a two-stage dye exclusion cytotoxicity assay. No killing of B10 spleen cell targets was seen with this reagent (cytotoxic titre <1/10). A/Sn spleen cell targets were efficiently lysed (titre of >1/640 with 50% maximum cytotoxicity). In tail skin preparations from chimaeras, the ratio of transplantation antigens (K^b) to Ia molecules is altered compared with normal tail skin preparations[10] (*a*). In chimaeras, there are fewer Ia molecules relative to transplantation antigens. The reason for this altered ratio is not clear. It seems likely that it represents the partial repopulation of epidermal Ia-bearing cells from bone marrow precursors. The monoclonal antibody used in *b* always gives smaller nonspecific peaks than immunoprecipitations using alloantisera. See Fig. 1 legend for explanation of markers.

Differential sensitivity of immunofluorescence and cytotoxicity assays, or bystander effects in the cytotoxic assays or contaminating Sk antibody might explain the conflicting cytotoxicity[6–8] and immunofluorescence[11,12,14,15] data. At present, we feel the simplest interpretation of our data is that the Ia molecules present in the epidermis are synthesised by bone marrow-derived cells, probably the Langerhans cells.

Phagocytic macrophage cells are involved in the presentation of antigen to T cells. The subsequent activation and proliferation of the T cells involves the Ia molecules on the antigen-presenting macrophage[19]. In guinea pigs, Langerhans cells can function both as stimulators in a mixed lymphocyte reaction and as antigen-presenting cells[16]. Perhaps Langerhans cells in the epidermis function like macrophages[18]. A high concentration of antigen-presenting cells in the skin is reasonable, as the skin is the first line of defence against pathogens. It was suggested that non-lymphoid Ia-positive epithelial tissues also might be immunologically relevant[13]. The precise immunological implications of Ia molecules on cells in the epidermis are unknown. However, the observations reported here are intriguing because they clearly raise the possibility that Ia molecules may be restricted to cells involved in the immune response.

We thank Glen Matsushima for technical assistance in antiserum production, Marilyn Kehry for donating the rabbit anti-MOPC 104E (Rαμ), Dr Len Herzenberg for providing the anti-I-A^k-producing hybridoma through the Salk Institute, and Mitch Kronenberg for helpful discussions. J.G.F. is supported by a National Research Service Award (5 T32 GM07616), and J.A.F. is the recipient of American Cancer Society Faculty Research Award FRA-179. This research is supported by NIH grants AI 10781 and CA 22662.

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1. Klein, J. *Biology of the Mouse Histocompatibility-2 Complex*, 389–489 (Springer, New York, 1975).
2. Shreffler, D. C. & David, C. S. *Adv. Immun.* **20**, 125–195 (1975).
3. Murphy, D. B., Herzenberg, L. A., Okumura, K., Herzenberg, L. A. & McDevitt, H. O. *J. exp. Med.* **144**, 699–712 (1976).
4. Hauptfeld, V., Hauptfeld, M. & Klein, J. *J. Immun.* **113**, 181–188 (1974).
5. Colombani, J., Colombani, M., Shreffler, D. C. & David, C. *Tissue Antigens* **7**, 74–85 (1976).
6. Hammerling, G. J., Mauve, G., Goldberg, E. & McDevitt, H. O. *Immunogenetics* **1**, 428–437 (1975).
7. Klein, J., Geib, R., Chiang, C. & Hauptfeld, V. *J. exp. Med.* **143**, 1439–1452 (1976).
8. Kreo, C. J., Steinmuller, D. & David, C. S. *Cell. Immun.* **46**, 239–246 (1979).
9. Delovitch, T & McDevitt, H. O. *Immunogenetics* **2**, 39–52 (1975).
10. Frelinger, J. G., Wettstein, P. J., Frelinger, J. A. & Hood, L. *Immunogenetics* **6**, 125–135 (1978).
11. Klareskog, L., Tjerlund, U. M., Forsum, U. & Peterson, P. A. *Nature* **268**, 248–250 (1977).
12. Rowden, G., Lewis, M. G. & Sullivan, A. K. *Nature* **268**, 247–248 (1977).
13. Winman, K. *et al. Nature* **276**, 711–713 (1977).
14. Stingl, G., Katz, S. I., Shevach, E. M., Wolff-Schreiner, E. & Green, I. *J. Immun.* **120**, 570–578 (1978).
15. Rowden, G., Phillips, T. M. & Delovitch, T. L. *Immunogenetics* **7**, 465–478 (1978).
16. Stingl, G., Katz, S. I., Clement, L., Green, I. & Shevach, E. M. *J. Immun.* **121**, 2005–2013 (1978).
17. Prunieras, M. *J. invest. Derm.* **52**, 1–17 (1969).
18. Silderberg-Sinakin, I., Thorbecke, G. J., Baer, R. L., Rosenthal, S. A. & Berezonsky, V. *Cell. Immun.* **25**, 137–151 (1976).
19. Schwartz, R. A., Yano, A., Stimpfling, J. H. & Paul, W. E. *J. exp. Med.* **149**, 40–57 (1979).
21. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. in *Current Topics in Microbiology and Immunology* Vol. V, 81 (Springer, New York, 1978).
20. Von Boehmer, H., Sprent, J. & Nabholz, M. *J. exp. Med.* **141**, 322–334 (1975).

Chapter IV

Peptide Map Comparisons of Epidermal and Spleen H-2 Molecules

The manuscript contained in this chapter is being submitted to

Immunogenetics for publication

Peptide Map Comparisons of Epidermal and Spleen H-2 Molecules

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Abstract. Peptide map comparisons of molecules encoded in the mouse H-2 complex isolated from epidermal cell preparations have been carried out. We previously showed that the Ia molecules from both the I-A and I-E subregion are synthesized by non-lymphoid bone marrow derived cells, probably Langerhans cells. The K and D or transplantation molecules are synthesized by both "true" epidermal cells and by non-lymphoid bone marrow derived cells. The tryptic maps generated by separating tryptic peptides by high pressure liquid chromatography (HPLC) of epidermal H-2 molecules are identical to their spleen cell counterparts. The biological significance of this finding is discussed.

Introduction

The major histocompatibility complex (MHC) of vertebrates has become a focal point for many investigations of the immune system. The murine MHC or H-2 complex consists of several regions (K, I, S, D, L, and perhaps TLa). Genes in the I region are involved in regulating the immune response to antigens, graft versus host reactivity, T-B cell collaboration, and graft rejection (Shreffler et al. 1976, Klein 1975). The I region also codes for serological detectable cell surface antigens, the Ia antigens. The I region has been further subdivided into several subregions (I-A, I-B, I-J, I-E, and I-C) by serological analysis and functional studies (Shreffler et al. 1976, Murphy et al. 1976). The Ia antigens isolated from detergent solubilized spleen cells and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis are composed of at least two subunits, the α and β polypeptides. The Ia antigens have a much more restricted tissue distribution than the K or D antigens which are present on virtually every tissue. In mice, the Ia antigens have been detected on B lymphocytes, macrophages, a subpopulation of T lymphocytes, fetal liver, spermatozoa (reviewed by Möller 1976), neutrophils (Okuda et al. 1979), epidermal cells (Hämmerling et al. 1975), dendritic cells (Steinmuller 1980) and thymic epithelial cells (Rouse et al. 1979). They are not found on a variety of other tissues (Hauptfeld et al. 1974, Colombani et al. 1976). The presence of Ia molecules on epidermal cells was puzzling. With the possible exception of spermatozoa, it is the only tissue not intimately involved in the immune response which expressed Ia determinants. Very careful immunofluorescent and immunoelectron-microscopic studies in mice demonstrate only the Langerhans cells ($\sim$3-5% of epidermal cells) react with anti-Ia sera (Rowden et al. 1978). This is in close agreement with results previously reported in humans (Rowden et al. 1977, Klareskog et al. 1977) and in guinea pigs (Stingl et al. 1978). It is difficult to relate these observations to to cytotoxicity studies (Hämmerling et

al. 1975, Klein et al. 1976, Krco et al. 1979) which indicate a majority of epidermal cells are Ia positive. Biochemical studies showed Ia molecules could be precipitated from a radiolabeled detergent solubilized epidermal cell extract by indirect immunoprecipitation using antisera directed against the entire I region (Delovitch and McDevitt 1975). In our previous experiments with epidermal cell preparations, we showed by immunoprecipitation that the Ia molecules from both the I-A and I-E subregions are synthesized by a non-B non-T bone marrow derived cell (Frelinger et al. 1978, Frelinger et al. 1979). It is likely that this cell is the macrophage-like Langerhans cell. We now demonstrate that the H-2 encoded molecules from two different normal tissues are in fact identical by peptide map criteria.

Materials and Methods

Mice and Sera. B10.HTT mice were raised at the mouse colony at the University of Southern California School of Medicine. Hyperimmune alloantisera were prepared by multiple injections of spleen, lymph node, and thymus cells as previously described (David et al. 1973). All alloantisera were characterized by direct cytotoxicity on a panel of inbred strains.

Preparation and Labeling of Cells. Spleen cells were labeled as described previously (McMillan et al. 1978). Briefly, single cell suspensions, made by teasing a spleen through a wire screen, were biosynthetically labeled at 2×10^7 nucleated cells/ml for 5 hours in Hanks Balanced Salt supplemented with 5% dialyzed fetal calf serum, 10 mM Hepes and 1 mCi ^{3}H-Tyr (specific activity 40 mCi/mmole). Epidermal cells were isolated by trypsinization as described previously (Scheid et al. 1972). They were labeled at 1×10^7 cells/ml. Spleen cell suspensions were lysed in a volume equal to the incubation mixture with 0.01 M Tris, 0.15 M NaCl, and 0.5% Triton X-100 at pH 7.4. Epidermal cells were lysed identically to spleen cells except at a concentration of 3×10^6 cells/ml.

Immunoprecipitations. The glycoprotein fraction was isolated by Lens culinaris affinity chromatography (Hayman and Crumpton 1972). This fraction was concentrated back to its original volume in a B-15 minicon (Amicon), and preprecipitated with Staphylococcus aureus Cowan I strain (Staph A) prepared according to Cullen and Schwartz (1976) to remove any nonspecifically binding material. The lectin-purified cell extracts were incubated with specific alloantisera, and subsequently precipitated with Staph A. The Staph A was washed 3 times, changing tubes for the last wash, and the bound material eluted with 2% SDS, 2% 2-mercaptoethanol, and 50 mM Tris, pH 6.8. The immunoprecipitated polypeptides were separated by preparative SDS polyacrylamide gel electrophoresis. The proteins were eluted in 0.01% SDS, mixed with 1 mg porcine immunoglobulin and lyophilized.

Peptide Maps. The proteins were reduced, alkylated, and digested with trypsin (Brown et al. 1974 as modified by McMillan et al. 1978). The tryptic peptides were analyzed on a DuPont 830 high pressure liquid chromatograph, and eluted with a gradient of acetone at 49°C (McMillian et al. 1978). We used a Zorbax CN column for the Ia molecules. A C18 column was used to separate tryptic peptides of the D^d molecule because it retained these peptides better. One ml fractions were dried and counted. The amount of radioactivity was determined on a Beckman LS 9000 scintillation counter. Tryptic peptides prepared from α-Lactalbumin reduced and then alkylated with ^{14}C-Iodoacetmide were the generous gift of Dr. Sandra Ewald, Montana State University at Bozeman. C^{14} labeled α-Lactalbumin tryptic peptides were separated using a chromo beads ion exchange column and eluted with a pyridine-acetate gradient (Brown et al. 1974).

Results

HPLC and Ion Exchange Separate Similar Number of Peptides. In this study, we used high pressure liquid chromatography (HPLC) to separate tryptic

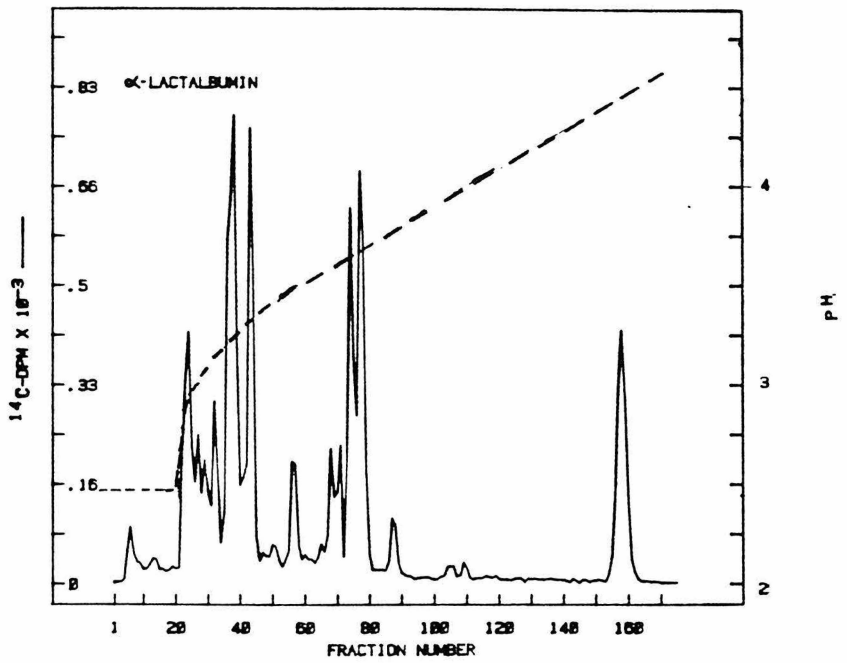
peptides to look for differences between molecules. Since resolution of the peptides was so critical, it was important to directly demonstrate that the separation of the peptides by HPLC was comparable to another commonly used technique, ion exchange chromatography. We have compared tryptic peptide separation for several molecules using conventional ion exchange chromatography and HPLC. As an example, we present the results for α-Lactalbumin in Fig. 1a and 1b. Ion exchange chromatography separates peptides primarily by their charge differences, whereas HPLC separates peptides primarily on the basis of their hydrophobicity. The separation of tryptic peptides is excellent using HPLC. The number of peptides separated by HPLC appears to equal the number separated by ion exchange chromatography (Fig. 1a,b), however, HPLC is much faster and more reproducible.

The D^d Polypeptide and β_2-Microglobulin from Spleen and Epidermal Cells are Identical by Peptide Maps. The D^d and β_2-microglobulin polypeptides were isolated by indirect immunoprecipitation using [A.SWxB10.A(1R)]F_1 anti B10.A serum from lentil lectin purified extracts of epidermal and spleen cells (see Table 1). Thereafter, the polypeptides from spleen and epidermis were treated identically. The tryptic peptides were separated by high pressure liquid chromatography. Except for some minor variations in intensity, the pattern of these peptides is identical, as shown in Fig. 2a and 2b.

The β_2-microglobulin peptide maps are informative in several respects. One would predict four tyrosine containing tryptic peptides, each with one tyrosine, from mouse β_2-microglobulin on the basis of homology to the known β_2-microglobulin sequences (Kabat et al. 1979). If one assumes the smallest peak is the result of a partial cleavage of the smallest major peak, the observed ratio of peaks is 0.95:0.93:0.92:1 in good agreement with the predicted ratio of 1:1:1:1. Thus HPLC appears to provide a quantitative recovery of each of the tryptic peptides. Further, the very clean separation of the five peptides demonstrates the practicality

Fig. 1. A comparison of tryptic peptide maps by ion exchange and high pressure liquid chromatography. Samples of C^{14}-labeled α-Lactalbumin tryptic peptides were separated by (a) HPLC and (b) ion exchange chromatography. (....) denotes the concentration of organic solvent; (----) denotes the pH gradient.

A)


α-LACTALBUMIN
.83
.66
.5
.33
.16
0
^{14}C-DPM X 10^{-3}
4
3
2
pH
1
20
40
60
80
100
120
140
160
FRACTION NUMBER

B)

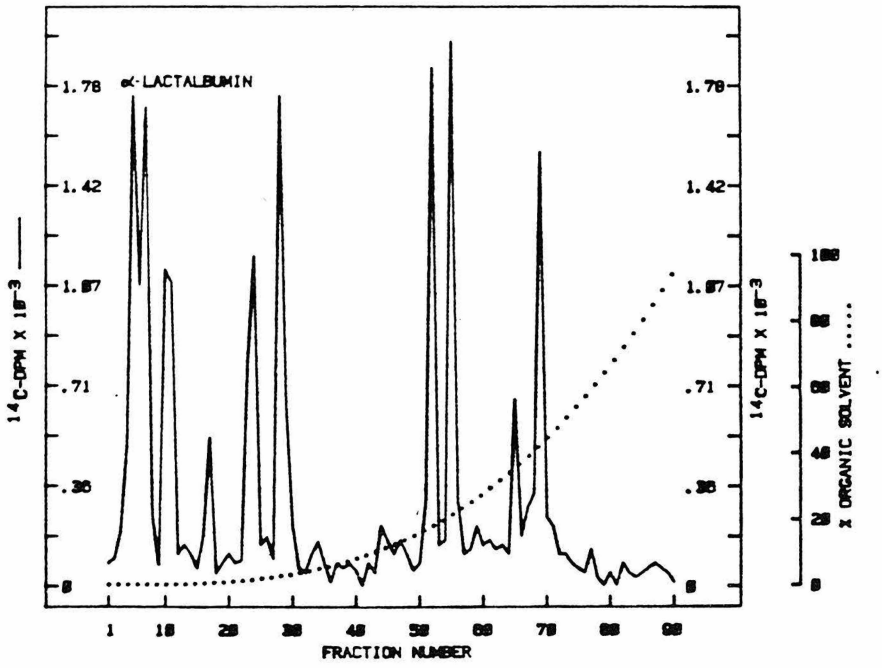

α-LACTALBUMIN
1.78
1.42
1.07
.71
.36
0
^{14}C-DPM X 10^{-3}
100
80
60
40
20
0
% ORGANIC SOLVENT .....
1
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 1

Table 1. The Antisera Used to Precipitate H-2 Molecules from B10.HTT Mice

Antisera	Relevant H-2 antigens when tested on B10.HTT targets
A.TH anti A.TL*	$I\text{-}E^k$, $I\text{-}C^k$
A.TL anti A.TH+	$I\text{-}A^s$, $I\text{-}B^s$, $I\text{-}J^s$
$[A.SWxB10.A(1R)]F_1$ anti B10.A	D^d

*Since Ia-like membrane antigens have not been demonstrated for the S region from lymphocytes, this antiserum is probably reacting principally with I-Region molecules.

+I-J is expressed on only a small subpopulation of lymphocytes and probably does not contribute to the observed reaction. Since no Ia specificities can be attributed to the I-B subregion and little compelling evidence can be produced for its existence, we have designated all reactions with I-A and I-B as I-A.

Fig. 2. Tryptic map comparisons of D^d and β_2-microglobulin polypeptides isolated from spleen and from epidermal cells. (a) Tryptic peptide maps from the D^d polypeptides. (——) denotes D^d from spleen; (----) denotes D^d from epidermal cells. (b) Tryptic peptide maps of β_2-microglobulin. (——) denotes β_2-microglobulin from spleen; (----) denotes β_2-microglobulin from epidermal cells. (....) denotes the concentration of organic solvent.

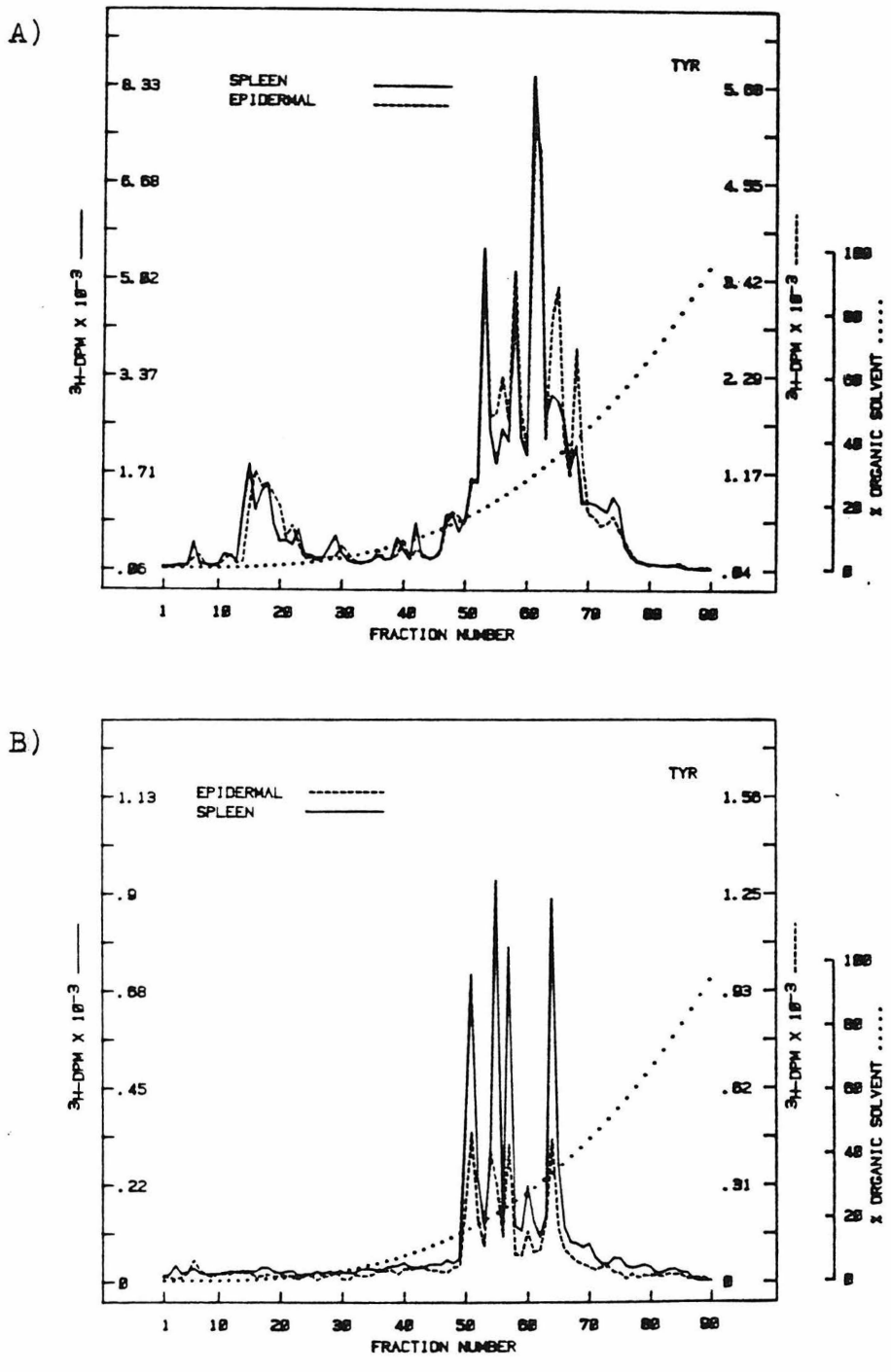

A)
SPLEEN
EPIDERMAL
TYR
8.33
6.68
5.82
3.37
1.71
.86
5.68
4.55
3.42
2.29
1.17
.84
^{3}H-DPM X 10^{-3} ——
^{3}H-DPM X 10^{-3} ------
% ORGANIC SOLVENT .....
1 10 20 30 40 50 60 70 80 90
FRACTION NUMBER
B)
EPIDERMAL
SPLEEN
TYR
1.13
.9
.68
.45
.22
0
1.58
1.25
.93
.62
.31
0
^{3}H-DPM X 10^{-3} ——
^{3}H-DPM X 10^{-3} ------
% ORGANIC SOLVENT .....
1 10 20 30 40 50 60 70 80 90
FRACTION NUMBER

Figure 2

of separating tryptic peptides of small molecules and thus should be useful in quick preparative separations (for example in the isolation of tryptic peptides of cyanogen bromide fragments).

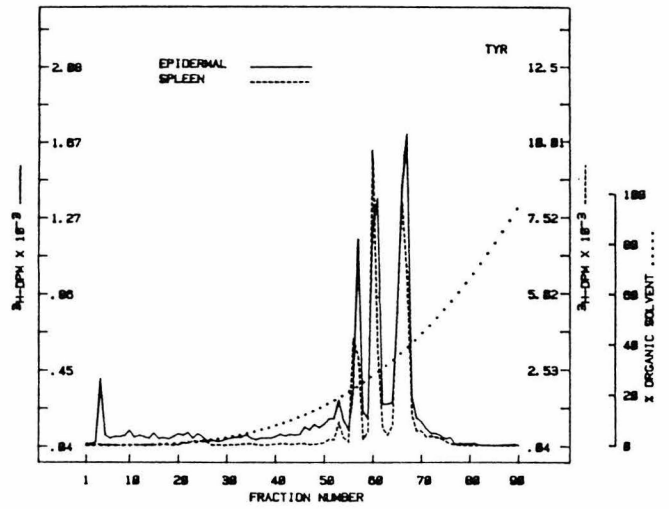
The Ia Polypeptides from Epidermal Cells and Spleen Cell Preparations Are Identical by Peptide Maps. Molecules encoded in the I-A subregion were immunoprecipitated from B10.HTT spleen and epidermal cell preparations using A.TL anti-A.TH serum (see Table 1). The tryptic peptides were again separated by HPLC. The A_α polypeptides are compared in Fig. 3a and the A_β polypeptides are compared in Fig. 3b. These patterns are indistinguishable. Polypeptides encoded in the I-E subregion were sequentially immunoprecipitated from B10.HTT mice using A.TH anti-A.TL (see Table 1). The yield of these molecules was much lower than for the I-A subregion molecules. Insufficient radioactivity was recovered in the E_β molecule for reliable peptide maps. The tryptic peptide maps of the E_α molecule from epidermal and spleen cells were identical (Fig. 3c).

Discussion

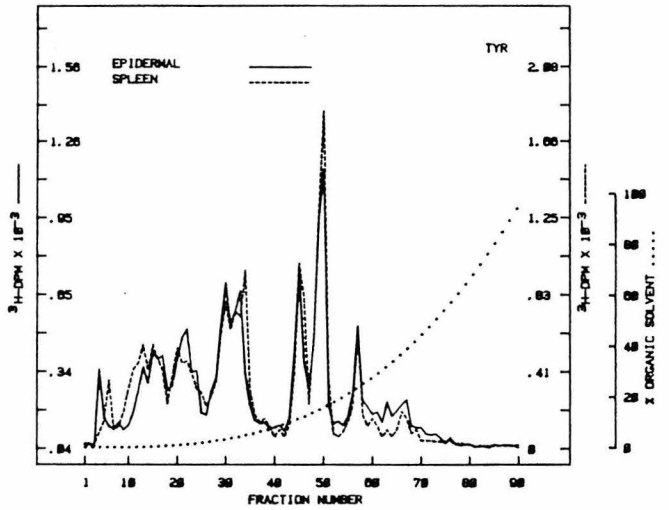
H-2 Polypeptides from Spleen and Epidermal Cells Are Identical by Peptide Maps. We have demonstrated that molecules isolated by indirect immunoprecipitation have identical tryptic peptide patterns on HPLC. We see no evidence for tissue polymorphism. We biosynthetically labeled with ^{3}H-tyrosine since many Ia molecules label poorly with ^{3}H-lysine and ^{3}H-arginine. Because we digested the molecules with trypsin which cleaves carboxy terminal to lysine and arginine residues, it is likely that not every tryptic peptide in these molecules contains a tyrosine and thus is labeled. However, peptide map analysis is a very sensitive technique which tends to overemphasize differences between molecules. Hence, these molecules must be extremely similar if not identical. The finding that anti-Ia sera raised by skin grafting is equivalent to anti-Ia sera raised by injection

Fig. 3. Tryptic map comparisons of I-A and I-E encoded polypeptides isolated from spleen and epidermal cells. (a) A_α polypeptides isolated from spleen and epidermal cells. (----) denotes spleen; (——) denotes epidermal molecules. (b) A_β polypeptides. (----) denotes spleen and (——) denotes epidermal molecules. (c) E_α molecules. (----) denotes spleen and (——) denotes epidermal molecules.

A)


TYR
EPIDERMAL
SPLEEN
FRACTION NUMBER
% ORGANIC SOLVENT

B)


TYR
EPIDERMAL
SPLEEN
FRACTION NUMBER
% ORGANIC SOLVENT

C)

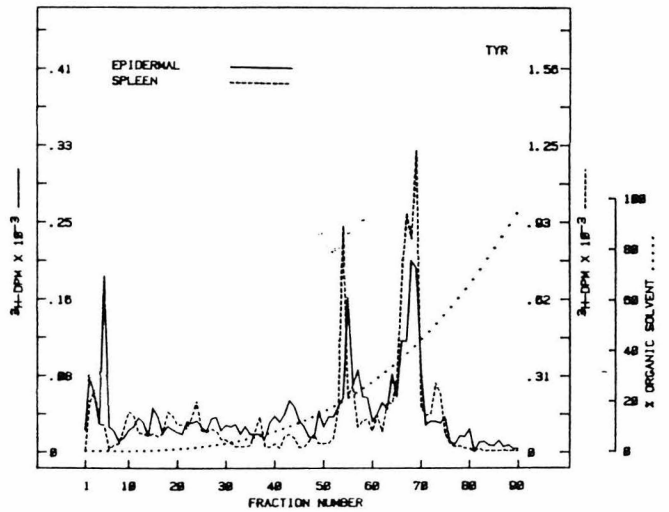

TYR
EPIDERMAL
SPLEEN
FRACTION NUMBER
% ORGANIC SOLVENT

Figure 3

of lymphoid cells is consistent with this conclusion (David et al. 1973, Sachs and Cone 1973).

No tissue polymorphism exists for the D^d or "transplantation" molecule. This direct chemical evidence supports the long held serological evidence that all cells in the mouse express identical K and D polypeptides.

Which Cells in the Epidermis Are the Source of the H-2 Molecules? We showed previously that the Ia molecules from epidermal preparation are not from T or B cells (Frelinger et al. 1978). Further, in long term chimeras utilizing antisera specific for either the donor or recipient Ia, we could only immunoprecipitate Ia molecules of the donor haplotype and not the recipient haplotype. Thus we concluded the Ia molecules are synthesized from a bone marrow derived cell, probably the Langerhans cell (Frelinger et al. 1979). In the same chimera experiments we precipitated a K or "transplantation" molecule of the recipient haplotype. Thus it is likely that both "true" epidermal cells (melanocytes, keratinocytes, etc.) and the non-lymphoid bone marrow derived cells contribute to the synthesis of these K or D "transplantation" molecules. The Ia molecules from spleen are most probably derived from B cells (Jones et al. 1978). The K or D molecules isolated from spleen cells are from a mixture of cells including macrophages, T, and B cells.

The Identical Peptide Maps of H-2 Encoded Molecules Have Interesting Implications for Function. Many investigators have examined malignant tumors or cell lines of varying cellular origins in an effort to compare the K or D polypeptides to their counterparts isolated from normal cells. Several laboratories have reported differences in these transplantation molecules, in particular, the appearance of new K or D molecules (reviewed by Parmiani et al. 1979). One explanation for these differences is that the K and D polypeptides from distinct normal tissues are different, i.e., fibroblast and lymphoid K and D polypeptides

may be different. We have presented data which indicate there is no tissue polymorphism of a transplantation molecule (D^d). This is particularly relevant, since this is one of the molecules reported to be inappropriately expressed on SJL (H-2^s) reticular sarcoma cells while the normal H-2^s K or D molecules are lost (Roman and Bonavida 1979).

Functional Implications of Identical Ia Polypeptides from B Cells and Langerhans Cells. We report a detailed characterization of non-lymphoid Ia molecules in the mouse. There appears to be no difference between the Ia molecules isolated from spleen and epidermal cell preparations by our peptide map criteria. It is likely that the bone marrow derived cell in the epidermis that synthesizes Ia is the Langerhans cell (Frelinger et al. 1979). These cells are bone marrow derived (Katz et al. 1979) and strongly Ia positive (Rowden et al. 1978, Krco et al. 1979). In guinea pigs, Langerhans cells strongly stimulate in mixed lymphocyte reactions (MLR) and can present antigen in a T cell proliferation assay. Further, biosynthetically labelled Ia could only be immunoprecipitated from a Langerhans cell-enriched fraction and not from a Langerhans cell-depleted fraction of epidermal cells (Stingl et al. 1978). Langerhans cells are probably a specialized tissue macrophage (Shelley and Juhlin 1976, Silberberg-Sinakin et al. 1977). Thus, we have shown that two distinct cell types, B cells and the macrophage-like Langerhans cells, have identical Ia molecules by peptide map criteria. This may indicate a common mechanism of action for the Ia molecules on these cells. For example, a single T-cell receptor could interact with either macrophage and/or B-cell Ia. It will be important to chemically characterize Ia molecules from other cells, such as T cells, for which there is serologic evidence for distinct Ia molecules.

References

Brown, J.L., Kato, K., Silver, J., and Nathenson, S.G.: Notable diversity in peptide composition of murine H-2K and H-2D alloantigens. Biochemistry 13:3174-3178, 1974

Colombani, J., Colombani, M., Shreffler, D.C., and David, C.: Separation of anti-Ia (I-Region associated antigens) from anti-H-2 antibodies in complex sera by absorption on blood platelets. Description of three new Ia specificities. Tissue Antigens 7:74-85, 1976

Cullen, S.E., and Schwartz, B.D.: An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing Staphylococci. J. Immunol. 117:136-142, 1976

David, C.S., Shreffler, D.C., and Frelinger, J.A.: New lymphocyte antigen system (LNA) controlled by the Ir region of the mouse H-2 complex. Proc. Nat. Acad. Sci. USA 70:2509-2514, 1973

Delovitch, T., and McDevitt, H.O.: Isolation and characterization of murine Ia antigens. Immunogenetics 2:39-42, 1975

Frelinger, J.G., Hood, L., Hill, S., and Frelinger, J.A.: Mouse epidermal Ia molecules have a bone marrow origin. Nature 282:321-323, 1979

Frelinger, J.G., Wettstein, P.J., Frelinger, J.A., and Hood, L.: Epidermal Ia molecules from the I-A and I-EC subregion of the mouse H-2 complex. Immunogenetics 6:125-136, 1978

Hämmerling, G.J., Mauve, G., Goldberg, E., and McDevitt, H.O.: Tissue distribution of Ia antigens: Ia on spermatozoa, macrophages, and epidermal cells. Immunogenetics 1:428-437, 1975

Hauptfeld, V., Hauptfeld, M., and Klein, J.: Tissue distribution of I region associated antigens in the mouse. J. Immunol. 113:181-188, 1974

Hayman, M.J., and Crumpton, M.J.: Isolation of glycoproteins from pig lymphocyte plasma membrane using Lens culinaris phytohemagglutinin. Biochem. Biophys. Res. Commun. 47:923-930, 1972

Jones, P.P., Murphy, D.B., and McDevitt, H.O.: Identification of separate I region products by two-dimensional electrophoresis. In H.O. McDevitt (ed.) Ir Genes and Ia Antigens, pp. 203-213, Academic Press, New York, 1978

Kabat, E.A., Wu, T.T., and Bilofsky, H.: Sequences of Immunoglobulin Chains. U.S. Department of Health Education and Welfare, 1979

Katz, S.I., Tamaki, K., and Sachs, D.H.: Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature 282:324-326, 1979

Klareskog, L., Tjerlund, U.M., Forsum, U., and Peterson, P.A.: Epidermal Langerhans cells express Ia antigens. Nature 268:248-250, 1977

Klein, J.: Biology of the Mouse Histocompatibility-2 Complex, Springer-Verlag, New York, 1975

Klein, J., Geib, R., Chiang, C., and Hauptfeld, V.: Histocompatibility antigens controlled by the I region of the murine H-2 complex. I. Mapping of the H-2A and H-2C loci. J. Exp. Med. 143:1439-1452, 1976

Krco, C.J., Steinmuller, D., and David, C.S.: Expression of I region gene products on mouse tail epidermis cells. Cell. Immun. 46:239-246, 1979

McMillan, M., Cecka, J.M., Murphy, D.B., McDevitt, H.O., and Hood, L.: Peptide map analyses of murine Ia antigens of the I-E subregion using HPLC. Nature 277:663-664, 1979

Möller, G. (ed.): Biochemistry and biology of Ia antigens. Transplant. Rev. 30, 1976

Murphy, D.B., Herzenberg, L.A., Okumura, K., Herzenberg, L.A., and McDevitt, H.O.: A new I subregion (I-J) marked by a locus (Ia-4) controlling surface determinants on suppressor T lymphocytes. J. Exp. Med. 144:699-712, 1976

Okuda, K., Neely, B.C., and David, C.S.: Expression of H-2 and Ia antigens on mouse peritoneal Neutrophils. Transplantation 28:354-356, 1979

Parmiani, G., Carbone, G., Invernizzi, G., Pierotti, M., Sensi, M., Rogers, M., and Appella, E.: Alien histocompatibility antigens on tumor cells. Immunogenetics 9:1-24, 1979

Roman, J. M., and Bonavida, B.: Inappropriate alloantigen-like specificities detected on spontaneous and transplantable reticulum sarcomas of SJL/J mice. J. Immunogen. 7:61-70, 1980

Rouse, R.V., Van Ewijk, W., Jones, P.P., and Weissman, I.L.: Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122:2508-2515, 1979

Rowden, G., Phillips, T.M., and Delovitch, T.L.: Expression of Ia antigens by murine keratinizing epithelial Langerhans cells. Immunogenetics 7:465-478, 1978

Rowden, G., Lewis, M.G., and Sullivan, A.K.: Ia antigen expression on human epidermal Langerhans cells. Nature 268:247-248, 1977

Sachs, D., and Cone, J.L.: A mouse "B" cell alloantigen determined by gene(s) linked to the major histocompatibility complex. J. Exp. Med. 138:1289-1304, 1973

Scheid, M., Boyse, E.A., Carswell, E.A., and Old, L.J.: Serologically demonstratable alloantigens of mouse epidermal cells. J. Exp. Med. 135:938-955, 1972

Shelly, W.B., and Juhlin, L.: Langerhans cells form a reticuloepithelial trap for external contact antigens. Nature 261:46-47, 1976

Shreffler, D.C., David, C.S., Cullen, S.E., Frelinger, J.A., and Neiderhuber, J.E.: Serological and functional evidence for further subdivision of the I regions of the H-2 complex. In the XLI Cold Spring Harbor Symposium on Quantitative Biology, Origins of Lymphocyte Diversity, 41:477-487, 1976

Silberberg-Sinakin, I., Thorbecke, G.J., Baer, R.L., Rosenthal, S.A., and Berezowsky, V.: Antigen-bearing Langerhans cells in skin, dermal lymphatics and in lymph nodes. Cell. Immunol. 25:137-151, 1976

Steinmuller, D.: Passenger leukocytes and the immunogenecity of skin allografts. J. Inves. Dermatol., in press, 1980

Stingl, G., Katz, S.I., Clement, L., Green I., and Shevach, E.M.: Immunologic functions of Ia-bearing epidermal Langerhans cells. J. Immunol. 121:2005-2013, 1978

Chapter V

Structure of Ia Antigens from the Rat: Mouse Alloantisera Demonstrate At Least Two Distinct Molecular Species

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Structure of Ia antigens from the rat. Mouse alloantisera demonstrate at least two distinct molecular species*

Ia antigens isolated from spleen cells of rats and mice are composed of two polypeptide chains, designated α and β. Mouse alloantisera specific for the I-A^k and I-E^k subregions react with two distinct groups of rat Ia antigens, designated A-like and E-like, respectively. Two-dimensional gel electrophoresis and peptide map analysis demonstrate that the A-like antigens of rat are distinct from the E-like antigens. Both rat Ia antigens react with alloantiserum produced in rats congenic for the major histocompatibility complex (MHC). These results demonstrate for the first time that two distinct Ia antigens are present in the rat. Accordingly, the rat, like the mouse, may have Ia antigens encoded by at least two subregions of the rat MHC. The existence of multiple Ia gene products in rats is revealed by chemical techniques even in the absence of formal genetic evidence of more than one I subregion in the rat.

[I 2573]

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Abbreviations: **MHC:** Major histocompatibility complex **SaCI:** *Staphylococcus aureus* Cowan I **SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis **HPLC:** High-pressure liquid chromatography

1 Introduction

The major histocompatibility complex (MHC) of mammals is a genetic region which controls a variety of immunologically significant phenomena [1]. Detailed genetic, serological, and molecular analyses of the MHC of the mouse are available because of the availability of inbred, recombinant and congenic strains of mice. We are interested in employing chemical approaches to the characterization of MHC antigens from the rat in order to compare the genetic organization and homology relationships of the corresponding genes in rats and mice, two closely related rodent species.

In the rat, MHC is known as AgB [2], or H-1 [3]. Historically, knowledge of the genetic organization of the H-1 complex has been obtained by experiments testing hemagglutinating anti-

body production, skin graft rejection, graft *vs.* host reactions, mixed lymphocyte interactions occurring between experimentally manipulated inbred rats and chemical analysis of the MHC antigens. As recently as 1973 [4], there was no evidence for genetic subdivision of the H-1 complex. The assignment of genes controlling the level of the immune response to branched synthetic polypeptides (Ir genes) was made to H-1 in 1972 [5]. In 1977, evidence of recombination between the genes controlling mixed lymphocyte interactions (MLI) and the genes coding for the classical, serologically detected (SD) erythrocyte transplantation antigens was presented [6]. Since then, there have been several studies which have also indicated that Ir (and MLI) genes are separable from the genes encoding the SD antigens in the H-1 complex [7–9]. The recombination frequency between the Ir genes and the SD antigen genes is very low ($\leq 0.25\%$, J. Howard, personal communication). The production of congenic-resistant inbred strains of rats has greatly facilitated the study of the H-1 complex because specific alloantisera to this genetic region can be produced. Moreover, polypeptides with molecular weights similar to the transplantation antigens ($\geq 45\,000$ mol. wt.) and Ia polypeptides (α, 35 000 mol. wt.; β, 28 000 mol. wt.) of mice can be isolated from rat lymphocytes by indirect immunoprecipitation with these alloantisera [10, 11]. The partial N-terminal microsequence analysis of these putative transplantation antigens of rat reveals striking sequence homology to their mouse counterparts, and the presence of two discrete molecular species implies that the H-1 complex of rat like the H-2 complex of mouse has at least two genes encoding the transplantation antigens [11]. In the preceding report [12], we also demonstrate that the β polypeptides of the rat Ia antigens are heterogeneous in their amino acid sequences and potentially homologous to β chains encoded by both the I-A and I-E subregions of the mouse H-2 complex. Accordingly, the rat may have at least two discrete Ia antigens. Our early work and that of Sachs and his co-workers [10, 13] had demonstrated that mouse alloantisera could be used to precipitate antigens encoded by the rat H-1 complex. In this study, we employ mouse alloantisera specific for the I-A and I-E subregions to demonstrate by sequential immunoprecipitation, peptide map analysis, and two-dimensional gel electreophoresis that there are two distinct populations of rat Ia antigens.

2 Materials and methods

2.1 Rats

BN, BN.B2, BN.B4 rats are inbred strains, congenic except for the H-1 region, maintained at the Wistar Institute, Philadelphia, PA. The strains have been inbred for homozygosity at the H-1 complex following backcrossing and selection for the given H-1 haplotype for 11 generations. Lewis rats, inbred for many generations, were obtained from Microbiological Associates, Bethesda MD.

Table 1. Inbred strain combinations of rats used to prepare rat alloantiserum to H-1 antigens

Serum	Recipient strain	Donor strain	H-1 regions detected
R 150	(DA × BN.B2)F_1	BN	$H\text{-}1^n$
R 169	(LEW.BN × BN.B2)F_1	Lewis	$H\text{-}1^l$
R 143	(Lewis × BN.B2)F_1	DA	$H\text{-}1^a$
R 140	(BN.B4 × BN)F_1	BN.B2	$H\text{-}1^n$

Table 2. Inbred strain combinations of mice used to prepare mouse alloantisera to Ia antigens

Serum	Recipient strain	Donor strain	Mouse I regions detected[a]
031	(B10.T(6R) × B10.D2)F_1	B10.AQR	$ABJE^k$
064	(B10.S(7R) × A.CA)F_1	B10.HTT	EC^k
D3b	(C3H.H-2^o × 129)F_1	C3H	$ABJEC^k$
12	(A.TH × B10.HTT)F_1	A.TL	$AB^k(J^k)$
D33	(B10.D2 × A)F_1	B10.A(5R)	AB^b

a) Ia antigens have not yet been isolated from the I-B, I-J, and I-C regions.

2.2 Antisera

Antisera used in this study are listed in Tables 1 and 2. Mouse antisera 031 and 064 were a gift of Dr. H. O. McDevitt of Stanford University, and antiserum 12 was a gift of Dr. J. A. Frelinger of the University of Southern California. D3b and D33 were obtained from the National Institutes of Health, Bethesda, MD. All rat antisera were prepared at the Wistar Institute, Philadelphia.

2.3 Spleen cell preparations

Spleens were dissected from individual rats and minced through wire mesh to prepare single-cell suspensions. The cells were collected and washed in Hanks' buffered salt solution (HBSS) containing 2% fetal calf serum and 20 mM HEPES buffer. The lymphoid cells were then isolated on Ficoll-Isopaque gradients [14], washed, and counted. Approximately 2×10^7 live cells were incubated at 37 °C for 5 h in 1 ml of HEPES-HBSS-2% fetal calf serum containing 1 mCi (= 37 MBq) of [^{3}H]tyrosine (New England Nuclear, Boston MA). Cells were washed in ice-cold medium three times after this short-term culture and solubilized in ice-cold 0.01 M Tris/0.15M NaCl/0.25% Nonidet-P 40 (NP 40) buffer for 20 min. The insoluble material remaining after this treatment was removed by centrifugation, and the supernatant frozen until use. These preparations were standardized to 5×10^7 intact cells/ml.

2.4 Analysis of immune precipitates

Radiolabeled antigens were precleared with fixed *Staphylococcus aureus* Cowan I (SaCI) [15] to remove nonspecifically adhering material. Antiserum (usually 10 µl/10^6 cells in the lysate) was added to this precleared antigen preparation, and the mixture was incubated overnight at 4 °C. Immune complexes were removed from the mixture by adsorption to SaCI for 20 min on ice and pelleted by centrifugation. After washing the precipitates twice in cold Tris-NaCl-0.25% NP 40 buffer, the antigen-antibody complexes were eluted from the *S. aureus* by boiling for 3 min in 4% sodium dodecyl sulfate (SDS)-6 M urea. The eluates were collected by centrifugation, diluted with glycerol and buffer salts, reduced in 2% 2-mer-

captoethanol, and analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [16]. Gels were sliced and eluted and the radioactivity counted in a liquid scintillation counter.

2.5 Two-dimensional gel electrophoresis

Antigens were precipitated and isolated as above, except that the complexes were eluted from the SaCl with isoelectric focusing buffer [17]. Samples were than applied to non-equilibrium pH gradient gel electrophoresis exactly as described [17]. The first dimensional gels were incubated in SDS-containing reducing buffer and run on the second dimension of SDS-PAGE. Gels containing ^{3}H-labeled proteins were fixed in 3.5% perchloric acid [18] and prepared for subsequent fluorography [19]. Kodak XR-5 X-ray film was flashed with a strobe light before applying it to the dried gels. The autoradiograms in this report reflect an exposure period of approximately 25 days for gels containing labeled proteins from the $3 \times 10^6 - 5 \times 10^6$ cells originally immunoprecipitated.

2.6 Peptide map analysis using high-pressure liquid chromatography (HPLC)

Ia molecules were isolated as described above (Sect. 2.4). The proteins were eluted from the gels in 0.01% SDS mixed with 0.5 mg porcine immunoglobulin and lyophilized. The polypeptides were reduced, alkylated, and digested as described by McMillan et al. [20]. The polypeptides were boiled for 5 min in 0.5 ml of 0.6 M Tris, 0.02 M EDTA, 9% SDS, pH 8.3 buffer and reduced under argon for 3 h at 37 °C with dithiothreitol (0.025 M). The proteins were then alkylated in the dark for 1 h at room temperature using iodoacetamide (0.05 M), and reagents were then removed by centrifugation through a 2.5 ml column of Sephadex G-25 equilibrated with 0.05 M Tris, 2% 2-mercaptoethanol, 2% SDS, pH 7.5. The proteins were then precipitated with 20% trichloroacetic acid, washed, dried, and digested for 18 h at room temperature in 0.2 ml 0.05 M ammonium hydrogencarbonate using 10 µl of trypsin-TPCK (Worthington, Freehold, NJ; 10 mg/ml in 0.001 N HCl). Another 10 µl of trypsin solution was added and after 4 h the resulting peptides were lyophilized. The peptides were analyzed on a DuPont 830 HPL chromatograph using a 25 cm Zorbax-CN column at 49 °C. The column was initially equilibrated with 0.1 M sodium phosphate buffer, pH 2.1, and the peptides were eluted with a gradient of acetone. One-ml fractions were collected every minute directly into scintillation vials and evaporated to dryness; 0.5 ml 0.01% SDS and 4 ml Aquasol-2 (NEN, Boston, MA) were added to each vial, and the radioactivity was then determined on a Beckman LS 9000 scintillation counter.

3 Results

3.1 Rat alloantiserum detects typical MHC molecules

Rat transplantation antigens and Ia antigens are very similar in their molecular weights to the MHC proteins isolated from mouse spleen cells [12]. Essentially the same gel patterns are found for H-1 complex antigens from four rat strains (BN, BN.B4, BN.B2, and Lewis). These observations suggest that the rat alloantisera, listed in Table 1, detect primarily MHC antigens.

3.2 Mouse alloantisera react with Ia molecules from four different rat haplotypes

The antigens of the rat H-1 complex react with highly specific alloantisera prepared against mouse Ia antigens. Ia antigens from Lewis, BN, BN.B2, and BN.B4 rats react with antisera directed to the entire mouse I region, the I-A subregion and the I-E subregion. A summary of the various mouse alloantisera reacting with rat MHC antigens is presented in Table 2. Both the α and β components of rat Ia antigens are found in all of the immunoprecipitates obtained with mouse alloantisera. More extensive studies to characterize the nature of these cross-reactions were conducted on BN antigen preparations.

3.3 Mouse alloantisera react with the same rat MHC antigens as rat alloantisera

We have tested the reactivity of one mouse antiserum (D3b, which detects the whole I^k region in H-2^k mice) as it compares with the reactivity of the appropriate rat alloantiserum (Fig. 1). A soluble lysate of spleen cells from BN rats was treated with (DA × BN.B2)F_1 anti-BN antiserum until all of the serologically reactive molecules were removed. A mouse alloantiserum directed to the entire I^k region (D3b) detected no remaining BN antigens (Fig. 1C). This experiment indicates that the mouse anti-I^k reagent recognizes only Ia molecules that are detected by the congenic rat alloantiserum. Because the rat alloantisera appear to recognize predominantly MHC antigens, this observation suggests the mouse alloantisera also are reacting with antigens encoded by the rat MHC.

3.4 Mouse alloantisera specific for the Ia antigens of two different mouse haplotypes both react with the same Ia molecules of the rat

Mouse alloantisera specific for the I-A subregions of mice of the H-2^b [(B10.D2 × A)F_1 anti-B10.A(5R)] and H-2^k

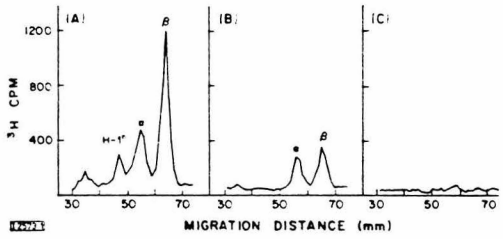

Figure 1. SDS-PAGE patterns of [^{3}H]tyrosine-labeled BN antigens. (A) BN antigens detected by R150 rat alloantiserum. (B) BN antigens detected by mouse anti-I^k alloantiserum (D3b). (C) BN antigens detected by mouse anti-I^k alloantiserum (D3b) after initial precipitation with R150 rat alloantiserum. The rat antigen preparations were precleared for immunoglobulins and other nonspecific peaks by preliminary precipitation with normal mouse serum and SaCl. The peak designated H-1^n contains BN major transplantation antigens. Ia antigens are labeled α and β.

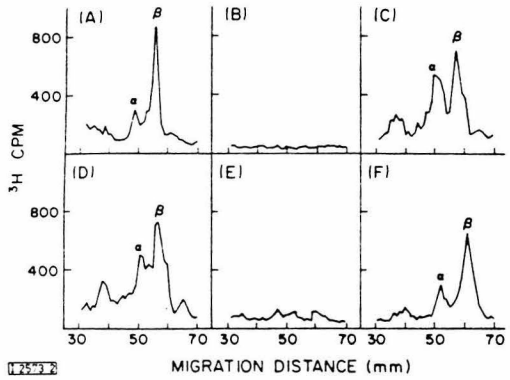

Figure 2. Sequential precipitations of [^{3}H]tyrosine-labeled BN Ia antigens. BN rat antigens were precipitated with mouse anti-I-E^k antiserum (A) until no further reactivity could be detected with this antiserum (B). The cleared supernatant from B was treated with mouse anti-I-A^k antiserum, to obtain the gel profile in (C). The reciprocal experiment is shown in panels (D-F), where anti-I-A^k antiserum was used as the first reagent (D), until all antigens reacting with this antiserum were depleted (E), and the supernatant from (E) was then precipitated with mouse anti-I-E^k antiserum (F). This experiment reveals that there are two serologically separable Ia antigens in the BN rat spleen cell preparation.

[(A.TH × B10.HTT)F$_1$ anti-A.TL] haplotypes were each employed to exhaustively precipitate two aliquots of spleen cell antigens from the BN rat. When the supernatants of these reactions failed to react with the homologous reagent, they were reacted with the reciprocal antiserum. In each case, the second mouse alloantiserum failed to precipitate any additional rat Ia molecules. Accordingly, the same rat Ia molecules must contain serological specificities that cross-react with mouse alloantisera to the I-A subregion of two distinct haplotypes, H-2^b and H-2^k.

3.5 Two mouse alloantisera specific for the I-A and I-E subregions, respectively, react with two distinct populations of rat Ia molecules

Two aliquots of BN spleen cell preparations were precipitated with either anti-I-A^k or anti-I-E^k antiserum (see Table 2). The supernatants of each aliquot were checked for residual activity with these reagents until all precipitable material was cleared from the antigen preparation (Fig. 2). These cleared supernatants were then treated with the opposite reagent, and the resulting immune precipitates were analyzed by SDS-PAGE. The mouse anti-I-A^k antiserum recognizes rat Ia molecules that fail to react with the mouse anti-I-E^k antiserum. Likewise, the anti-I-E^k antiserum recognizes rat Ia molecules that fail to react with the anti-I-A^k antigens. Therefore, the mouse alloan-

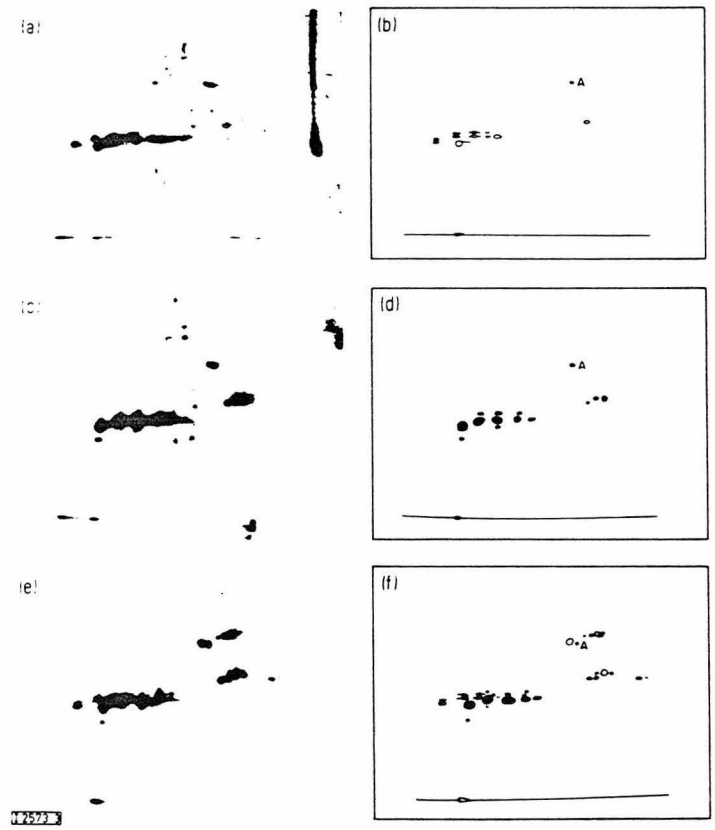

Figure 3. Two-dimensional gel pattern of [^{3}H]tyrosine-labeled BN (H-1^n) antigens. Nonequilibrium pH gradient electrophoresis (NEPHGE) of immunoprecipitated BN antigens was performed in the first (horizontal) dimension, followed by mol. wt. separation on SDS-PAGE gels in the second (vertical) dimension. The basic end of the NEPHGE gel is on the left, and the pH range is from 4.4 to 8.4 on each of the gels. Autoradiographs of the gels are shown on the left; schematic diagrams of the labeled MHC proteins are shown on the right. (a, b) BN molecules precipitated with mouse anti-I-A^k serum; (c, d) BN molecules precipitated with mouse anti-I-E^k serum; (e, f) BN molecules precipitated with rat alloantiserum to the whole H-1^n region (R150). The contaminating actin spot is indicated by the letter "A". In addition to the Ia antigen spots, the rat alloantiserum (e, f) recognizes the BN transplantation antigens (45000 mol. wt.), which appear in the vicinity of the actin spot. β_2-Microglobulin runs at the dye front.

tisera specific for the I-A and I-E subregions appear to react with two distinct populations of rat Ia molecules.

3.6 Two-dimensional polyacrylamide gel analyses of rat Ia molecules isolated with mouse anti-I-A and mouse anti-I-E reagents demonstrate two distinct populations of β polypeptides

Since the Ia molecules are comprised of two distinct polypeptides, α and β, it was of interest to determine whether one or both chains differed in the rat Ia molecules precipitated with either the mouse I-A or I-E alloantisera. To approach this question, we carried out the immunoprecipitation procedure on BN spleen cells employing three reagents: rat anti-H-1^n alloantiserum (Table 1), mouse anti-I-A^k antiserum and mouse anti-I-E^k antiserum (Table 2). These immunoprecipitates were submitted to two-dimensional gel electrophoresis (Fig. 3). Each of the three reagents yields immunoprecipitates with a complex pattern of α and β polypeptides. These complex patterns are similar to those seen in a comparable analysis of mouse Ia antigens [21], and the complexity of polypeptide spots appears to be due at least in part to different levels of glycosylation. There are several striking features of these data.

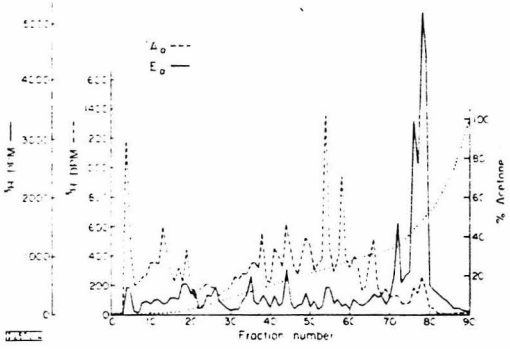

Figure 4. Tryptic peptide maps using HPLC. Tryptic peptides of α Ia molecules labeled with [^{3}H]tyrosine isolated using anti-I-A region antisera (– – –) and anti-I-E region antisera (——).

(a) The β polypeptides from the rat I-E-like molecules are distinct from those of the rat I-A-like molecules. Thus, we can tentatively conclude that there are two separate groups of rat β chains which we will designate A_β and E_β, respectively. (b) The pool of β polypeptides isolated by the rat alloantiserum appears to be equivalent to the sum of the pools of β polypeptides isolated by the two mouse alloantisera. Thus, the rat alloantiserum reacts with the two types of rat Ia molecules (*i.e.* I-A and I-E), but presumably it does not react significantly with additional molecular species. (c) The rat alloantiserum precipitates two groups of acidic spots which differ slightly in charge and mol. wt. These acidic spots, which are difficult to see in Fig. 3, but have been observed in repeated gels, are likely to be the rat α polypeptides on the basis of their mol. wts. The higher mol. wt. and less acidic group of putative α spots is clearly precipitated by the mouse anti-I-E^k reagent. However, we have been unable to make a clear subregion distinction between the acidic spots on two-dimensional gels using the mouse alloantisera due to the relative faintness of the α as compared to the β spots.

3.7 Tryptic peptide map analyses of rat Ia molecules isolated with mouse anti-I-A and mouse anti-I-E reagents demonstrate two distinct populations of α polypeptides

Rat α polypeptides from Ia molecules were isolated from [^{3}H]tyrosine-labeled BN spleen cell preparations using mouse alloantisera to the I-A^k and I-E^k products and preparative SDS-PAGE. The α polypeptides precipitated with each of these reagents were separately digested with trypsin, and the peptides were analyzed by HPLC as previously described [20]. The resulting peptide maps show that the α polypeptide precipitated with the anti-I-A^k mouse alloantiserum is clearly different from the α polypeptide precipitated with the anti-I-E^k mouse alloantiserum (Fig. 4). Furthermore, the elution pattern of the tyrosine-containing tryptic peptides of the anti-I-E-precipitated rat α chain is very reminiscent of the elution patterns of similar tryptic peptide maps of the mouse I-E α polypeptides. The tyrosine-containing peptides of the I-E-like α are very hydrophobic as are those of the mouse E^k_α and E^d_α polypeptides [20]. In fact, the major[^{3}H]tyrosine-containing peptides of the rat E_α-like polypeptide elute at identical positions in HPLC gradient to those of the mouse suggesting that these polypeptides are very homologous. The HPLC distribution of tryptic peptides from the rat α-like polypeptide, isolated using the mouse anti-I-A^k antiserum, is not so similar to those of the mouse A_α polypeptide by this analysis. The β-like molecules of rat are different from one another and are more hydrophilic relative to the α-like molecules of rat. These general features also are shared by mouse α and β polypeptides (data not shown).

4 Discussion

4.1 Ia molecules of the rat and mouse share many general features

The prevalent models for the organization of the MHC of various species rely heavily on similarity to, or dissimilarity from, the mouse H-2 system [1]. In the rat, Ir gene functions are found to be linked to the MHC, and Ia-like antigens are found to be encoded by this region as well. The direction of our efforts in this work has been to determine the number of antigens encoded by the rat MHC and assess their chemical characteristics. Both rat and mouse Ia molecules are comprised of two noncovalently associated polypeptides, the α about 35 000 in mol. wt. and the β about 28 000 in mol. wt. Partial N-terminal analyses reveal that there appears to be a single molecular species of rat α polypeptide which is homologous to its mouse E_α counterpart [12]. The Ia molecules of the rat and mouse also share serological specificities in that specific mouse alloantisera for the I region precipitate rat Ia antigens (this report [10, 11, 13]). Moreover, mouse alloantisera to the I-A and I-E subregions precipitate two distinct groups of Ia molecules from lymphoid cells of both the rat and the mouse. This observation is consistent with the fact the rat β chain appears to have at least two distinct sequences homologous to their mouse A_β and E_β counterparts [12]. Accordingly, the Ia antigens of the rat may be defined by virtue of their homology relationships to mouse Ia antigens at several different levels: subunit mol. wt. N-terminal amino acid sequence homology, reactivity to homologous alloantisera, and cross-reactivity with highly specific mouse alloantisera to the entire I region and to the I-A and I-E subregions.

4.2 The Ia molecules of the rat appear to be encoded by at least two distinct genes in the H-1 complex

The sequential precipitation of rat Ia antigens with the mouse anti-I-A and anti-I-E alloantisera indicates that the cross-reactive determinants reside on different molecules. In addition to the serological distinction, these rat Ia antigens are chemically distinct in terms of their migration on two-dimensional gels (β polypeptides) and tyrosine-containing tryptic peptide maps (α polypeptides). Thus, by analogy to the mouse, the rat I region equivalent (H-1 B) appears to code for at least two distinct Ia antigens, equivalent to those encoded by the I-A and I-E subregions in the mouse. Several additional lines of evidence suggest that each of the Ia β genes are encoded in the rat H-1 complex. (a) The rat I-A-like and I-E-like molecules can both react completely with congenic rat alloantiserum, presumably directed only against gene products of the MHC. (b) The β polypeptides of congenic rat strains appear to differ by possible haplotype-associated residues in the partial N-terminal sequences [12] and by distinct patterns with two-dimensional gel electrophoresis (data not shown). Since the congenic rats are presumably identical in their genetic constitutions but for the H-1 complex, this implies that the genes controlling the expression of rat β polypeptides (structural or regulatory) are encoded in the H-1 complex.

The rat Ia α polypeptides clearly differ from one another in the BN rat as shown by peptide map analysis. In addition, at least one of the two α polypeptides shows striking N-terminal sequence homology with the mouse I-E α polypeptide and the human Ia α polypeptide [12]. We have not yet determined whether the rat α polypeptides vary between haplotypes or whether there is sequence homology between the α molecule precipitated with mouse anti-I-A alloantiserum and the mouse I-A subregion α polypeptide. These studies are currently in progress.

4.3 The serological cross-reactions between the Ia antigens of rat and mouse pose an interesting evolutionary problem

The existence of cross-reactions between rat and mouse membrane antigens is not surprising. Studies of cross-reactions between the mouse thymus cell antigen, Thy-1, and the rat homologue of this antigen showed that the rat antigen could be detected by specific mouse alloantiserum which had been prepared to one allelic product of the mouse Thy-1 locus [22]. Sachs and his co-workers have presented evidence of Ia specificities shared by rats and mice, using mouse anti-rat antiserum and congenic mouse alloantiserum to the whole H-2 region [10, 13].

The cross-reactions appear to involve similar but not identical determinants on the Ia antigens of rat and mouse. The relative avidity of the mouse alloantibodies appears to be lower for rat than for mouse antigen preparations. For example, the Ia antigens of rat cannot be completely precipitated by repeated reactions with mouse anti-I^k antisera. However, the two-dimensional gel electrophoresis patterns of rat Ia molecules obtained by mouse alloantiserum are identical. Since the mouse alloantiserum reacts completely with homologous antigens, this implies a lower avidity for the rat antigens.

The interesting evolutionary problem posed by these studies is as follows. The mouse alloantisera are capable of distinguishing mice of one haplotype from those of a second, yet a mouse alloantiserum to the I region molecules of the $H\text{-}2^k$ haplotype reacts with rats of four different haplotypes. This indicates that at least some of the serological specificities which characterize mouse Ia molecules of the $H\text{-}2^k$ haplotype are present on rat Ia molecules of several different haplotypes. Moreover, the mouse alloantiserum to Ia molecules of the $H\text{-}2^b$ haplotype recognizes precisely the same molecules as mouse anti-I^k serum. This implies that the rat Ia molecules from many different haplotypes share at least some of the specificities characteristic of Ia antigens from mice of the $H\text{-}2^b$ haplotype. The results of Sachs et al. [10, 13] are similar to our own in that all of the mouse antisera which react with Ia antigens from one rat strain also react with Ia molecules from other rat haplotypes. Various genetic models have been discussed in an attempt to explain the existence of sets of antigenic determinants which are polymorphic within a species yet conserved between members of two different species [13, 23]. At the chemical level, the simplest explanation for these results would be that the alloantisera recognize a very limited number of determinants grouped on a very specific part of the antigen. Haplotype specificity would then be conferred by prohibitive amino acid substitutions within the epitope (*i.e.* by charge change or by the substitution of a large side chain) which interfere with the antibody binding. Antigenically neutral substitutions occurring within this same epitope in other species could allow binding of either haplotype-defining antibody.

It is interesting that even across a species barrier the mouse alloantisera seem to distinguish A-ness and E-ness. This would seem to imply a conservation of structure in the vicinity of the alloantigenic determinants (at least) which differentiates the two subregion products in the mouse. Since A-ness and E-ness seem also to be separate in the rat, one is lead to postulate that the separation of I-A and I-E occurred prior to the separation of mouse and rat. If so, cross-reactions between mouse alloantisera and Ia antigens of other species is a powerful probe for examining the expression of Ia antigens in species for which defined genetic systems are lacking.

Definition of the genetic organization responsible for the expression of two Ia antigens in the rat will require characterization of the cross-reactive molecules in other rat strains. We have demonstrated, for the first time, the existence of multiple and distinct rat Ia antigens. We hope that study of the rat Ia antigens will lead us to a greater understanding of the biochemical nature and functions of the Ia antigens in general.

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5 References

1 Götze, D. (Ed.), *The Major Histocompatibility System in Man and Animals*, Springer Verlag, New York 1977.
2 Elkins, W. L. and Palm, J., *Ann. N. Y. Acad. Sci.* 1966. *129:* 573.
3 Kren, V., Štark, O., Bûlá, V., Frenzl, B., Krenová, D. and Krsiaková, M., *Transplant. Proc.* 1973. *5:* 1463.
4 Palm, J. and Wilson, D. B., *Transplant. Proc.* 1973. *5:* 1573.
5 Günther, E., Rüde, R. and Štark, O., *Eur. J. Immunol.* 1972. *2:* 151.
6 Williams, R. M. and Moore, M. J., *Transplant. Proc.* 1977. *9:* 563.
7 Cramer, D.V., Shonnard, J. W. and Gill, T. J., III, *J. Immunogenetics* 1974. *1:* 421.
8 Štark, O., Günther, E., Kohoutová, M. and Vojcik, V., *Immunogenetics* 1977. *5:* 183.

9 Gunther, E., Štark, O. and Koch, C., *Eur. J. Immunol.* 1978. *8*: 206.
10 Shinohara, N., Cullen, S. E. and Sachs, D. H., *J. Immunol.* 1977. *118*: 2083.
11 Blankenhorn, E. P., Cecka, J. M., Götze, D. and Hood, L., *Nature* 1978. *274*: 90.
12 Cecka, J. M., Blankenhorn, E. P., Götze, D. and Hood, L., *Eur. J. Immunol.* 1980. *10*: 140.
13 Sachs, D. H., Humphrey, G. W. and Lunney, J. K., *J. Exp. Med.* 1977. *146*: 381.
14 Thorsby, E. and Bratlie, A., in Terasaki, P. I., (Ed.), *Histocompatibility Testing*. Munksgaard, Copenhagen 1970, p. 655.
15 Cullen, S. E. and Schwartz, B. D., *J. Immunol.* 1976. *117*: 136.
16 Laemmli, U. K., *Nature* 1970. *227*: 680.
17 O'Farrell, P. Z., Goodmann, H. M. and O'Farrell, P. H., *Cell* 1977. *12*: 1133.
18 Reisner, A. G., Nemes, P. and Bucholtz, C., *Anal. Biochem.* 1975. *64*: 509.
19 Laskey, R. A. and Mills, A. D., *Eur. J. Biochem.* 1975. *56*: 335.
20 McMillan, M., Cecka, J. M., Murphy, D. B., McDevitt, H. O. and Hood, L., *Nature* 1979. *227*: 663.
21 Jones, P. P., *J. Exp. Med.* 1977. *146*: 1261.
22 Douglas, T. C., *J. Exp. Med.* 1972. *136*: 1054.
23 Bodmer, W. F., *Transplant. Proc.* 1973. *5*: 1471.

Chapter VI

Characterization of Rat Class II Molecules with Restricted Mouse Anti-Ia Sera

The manuscript contained in this chapter is being submitted to

Immunogenetics for publication

Characterization of Rat Class II Molecules with Restricted Mouse Anti-Ia Sera

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Characterization of RT1 using mouse antisera

Abstract. Murine anti-Ia sera were tested for their reactivity with rat peripheral lymphocytes. Extensive cross-reactions were observed in a complement dependent cytotoxicity assay. In the majority of strain combinations, these reactions were specific for B cells. We demonstrated by absorption analyses that: 1) the reaction was specific, 2) B cells from all rat strains reacted with anti-I-E^k serum, and 3) the anti-I-A sera defined five new specificities. These antisera can also immunoprecipitate class II (Ia-like) molecules from radiolabeled detergent solubilized cell extracts. The rat equivalents of molecules encoded in the I-A and I-E subregions in mice can be isolated from all rat strains studied. The α and β subunits of the rat class II molecules were polymorphic when analyzed by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis. This observation allows the characterization of rat alloantisera of unknown specificity by comparing them to the defined reactivity of the mouse anti-Ia sera.

Introduction

The major histocompatibility complex (MHC) consists of tightly linked genes involved in regulating the immune response and has been identified in all mammalian species studied. The H-2 complex of the mouse has served as the prototype for identification of MHC class I and class II genes. Class I gene products H-2K, D, L are expressed in all tissues (reviewed in Klein 1975) whereas molecules encoded by the class II genes, I-A and I-E, are expressed primarily in B cells (Sachs and Cone 1973) and macrophages (Hämmerling et al. 1974). The identification of multiple H-2 class I and II genes has been facilitated by the selection of intra-H-2 recombinants.

Compared to the relatively advanced stage of the analysis of H-2 genes and their antigenic products, little is known about the genetics and biochemistry of the rat MHC (RT1). The existence of the RT1 (AgB) complex was first demonstrated serologically and presumably detected class I antigens (Bogden and Aptekman 1960; Kren 1960). The class II antigen(s) was detected originally as mixed lymphocyte culture (MLC) stimulating antigen(s) (Silvers et al. 1967). More recently, serological and biochemical analyses demonstrated the existence of two class I and two class II genes in RT1 (Shinohara et al. 1978; Blankenhorn et al. 1978; Sporer et al. 1979; Blankenhorn et al. 1980). However, a scarcity of recombinants within the class I and class II regions has limited the serological and biochemical analyses of individual class I and II molecules. Serological detection of single molecules would facilitate such an analysis.

Experiments by several groups (Sachs et al. 1977; Shinohara et al. 1978; Blankenhorn et al. 1980) detecting RT1 class II antigens with mouse anti-Ia sera suggest a fruitful approach to a more detailed characterization of individual RT1 class II alloantigens. Accordingly, we have analyzed the reactivity of restricted mouse anti-I-A sera on rat lymphocytes.

In this communication we report detailed serological and biochemical analyses of the reactivity of these antisera with rat class II molecules. These results directly demonstrate by serologic and biochemical criteria polymorphism for the rat class II molecules. The implications of these results for the further analysis of RT1 genes are presented.

Materials and Methods

Rats and Mice. The rats utilized in these experiments and their respective RT1 (AgB) haplotypes are described in Table 1. All rats were produced in our breeding colony at The Wistar Institute. Mice employed as positive and negative controls of antisera were of the same strains as the donors and recipients of the tested alloantisera; all mice were raised in our breeding colony at The Wistar Institute.

Alloantisera. The mouse alloantisera tested in this study were generously supplied by Dr. Jeffrey A. Frelinger, Department of Microbiology, University of Southern California Medical School, Los Angeles, California, and the NIH Alloantiserum Contract. The monoclonal antibody 10-2.16 was provided by Dr. Herzenberg through the Salk Institute, San Diego, California. The alloantisera and their specificities are presented in Table 2. Rat alloantisera specific for the Pta antigen expressed on peripheral rat T cells (Butcher and Howard 1977) were produced in this laboratory. Lewis anti-Fischer (anti-Pta 2) and BN.ALB-$RT1^b$ anti-Buffalo (anti-Pta 1) sera were produced by standard techniques (Götze et al. 1978). Lymph node cells not lysed by anti-Pta plus C' are 90-100% sensitive to anti-class II antisera and 90-100% surface Ig positive when tested with fluorescein conjugated polyvalent goat anti-rat Ig (Antibodies Incorporated, Davis, California).

Complement-Dependent Cytotoxicity. The complement-dependent cytotoxic test was a two-stage test performed as described by Frelinger et al. (1974),

Table 1. RT1 Haplotypes of Employed Rat Strains

Strain	RT1	(AgB)
Fischer	1	B1
BN.WF-RT1^{w}	w	B2
Lew.BN-RT1^{n}	n	B3
BN	n	B3
BN.DA-RT1^{a}	a	B4
DA	a	B4
August	c	B5
Buffalo	b	B6
MNR	m	B13

Table 2. Specificity of Tested Ia-Specific Mouse Antisera

Antiserum	H-2 region (+ other)	Ia specificity
A.TH anti-A.TL[a]	I^k	1,2,3,7
A.TH anti-A.TL (NIH)	I^k	1,2,3,7
(B10 x A.TH)F_1 anti-A.TL	I^k	1,2,7
A.TL anti-A.TH	$I\text{-}A^s$ (+ Tla^b)	4,5,9,12
(A.TL x B10.A(5R))F_1 anti-A.TH (NIH)	$I\text{-}A^s$	4,5,12
(A.TH x B10.HTT)F_1 anti-A.TL	$I\text{-}A^k$	1,2,3
(A.BY x B10.HTT)F_1 anti-A.TL (NIH)	$I\text{-}A^k$	1,2
(A x B10.D2)F_1 anti-B10.A(5R)	$K^bI\text{-}A^b$	8,9,21
CWB anti-C3H monoclonal 10-2.16	$I\text{-}A^k$	17
(B10 x HTI)F_1 anti-B10.A(5R) (NIH)	$I\text{-}E^k$	7,22

[a]Supplied from J. A. Frelinger unless otherwise noted.

[b]Possibility of anti-Qa-2 activity (Flaherty et al., 1977).

with minor modifications described previously (Götze et al. 1978). Rabbit serum, selected for low toxicity on rat lymphocytes, was the source of complement. Rabbit complement was essential for uniform detection of reactions of mouse alloantisera and rat lymphocytes.

Negative Selection of B Cells. T cells were eliminated from cervical lymph node cell preparations by mass depletion as described by Niederhuber et al. (1975). Briefly, lymph node cells were suspended by extrusion through fine mesh nylon bolting cloth. Twenty-five million viable cells were treated with 2.5 ml of anti-Pta serum diluted in medium 199 plus 5% fetal calf serum (FCS) by incubation at 37°C for 30 minutes. Lymphocytes were spun down and the diluted antiserum removed and replaced by 2.5 ml of rabbit complement diluted in 199. Complement-treated cells were incubated at 37°C for 30 minutes. The surviving cells were washed 3 times in 199 and 5% FCS and incubated in same at 37°C for 45 minutes to allow antiserum plus C'-damaged cells to undergo complete lysis. The cell suspensions were layered over Ficoll-Sodium metrizoate (Nyegaard & Co., Oslo, Norway) (p = 1.090) (Davidson and Parish 1975) and were spun at 2000 x g for 5 minutes to remove dead cells. The viable cells at the interface were removed, washed three times, and resuspended for testing.

Absorptions. Absorption of antisera with splenic lymphocytes was carried out according to the technique described by Götze et al. (1978) for red blood cell adsorption. Antiserum was diluted to the lowest concentration which resulted in maximum kill of targets from all rat strains. 'X' number of microliters of diluted antiserum was absorbed with 'X' x 10^6 splenic lymphocytes for 60 minutes at 37°C. Absorbed antiserum was diluted in doubling dilutions from 1/2 to 1/18 and delivered to microtiter plates for test. All absorptions with rat spleen cells were accompanied by unabsorbed controls and control samples absorbed with positive and negative mouse spleen cells.

Immunoprecipitation of RT1 Class II Antigens. Single cell suspensions were made from the spleens of individual rats. Red blood cells were lysed by $KHCO_3$-NH_4Cl treatment, and the remaining lymphocytes washed three times. Lymphocytes were cultured at 2×10^7 viable cells/ml in Hank's balanced salt solution supplemented with 5% dialyzed fetal calf serum, 10 mM HEPES, 1 mCi each of ^{3}H-tyrosine and leucine, and Minimal Eagle's Media vitamins. After 5 hours incubation at 37°C, the cells were washed twice in Hank's and then lysed in 2 ml of a solution of 0.5% Triton X-100, 10 mM Tris, 0.14 M NaCl pH 7.4 (TBS-TX-100) for 30 minutes on ice. Debris was removed by centrifugation. The supernatants containing the alloantigens were stored at -70°C.

Radiolabeled rat MHC molecules were isolated by indirect immunoprecipitation using Staphylococcus aureus (Staph A) as described by Cullen and Schwartz (1976) with minor modifications. Briefly, the TBS-TX-100 supernatants were precleared with 200 µl of washed Staph A and incubated with specific antisera for 2-4 hours at 4°C. Staph A was added for 1 hour on ice; the Staph was pelleted by centrifugation and washed three times in TBS-TX-100, changing tubes at the last wash. Proteins were eluted from the Staph A by boiling in a solution of 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, and 50 mM Tris pH 6.8. The Staph A was pelleted and the supernatant removed. Glycerol and pyronin Y were added to the samples which were electrophoresed on 10% polyacrylamide SDS slab or disc gels (Laemmli 1972). Disc gels were sliced into 1 mm segments and eluted overnight in 0.5 ml of 0.01% SDS in scintillation vials. Four ml of Aquasol-2 were added and amount of radioactivity was determined on a Beckman LS9000 scintillation counter. Slab gels were stained, destained, and prepared for fluorography (Laskey and Mills 1975). The dried gels were placed on preflashed Kodak XR-5 X-ray film which was developed after storage for 2-4 days at -70°C.

Results

Direct Complement-Dependent Cytotoxicity: Mouse Anti-Ia Sera Cross-React with Rat B Cells. The reactivity of a panel of mouse Ia-specific alloantisera with rat lymph node cells was determined. The three types of anti-Ia sera tested were: 1) reactive with I-A plus I-E antigens, e.g., A.TH anti-A.TL; 2) reactive with I-A antigens, e.g., (A.TH x B10.HTT)F_1 anti-A.TL; and 3) reactive with I-E antigens, e.g., (B10 x HTI)F_1 anti-B10.A(5R). The titers and maximum lysis values in these tests are presented in Table 3. As expected from preliminary experiments (P. Wettstein, unpublished data), no reactivity with class I antigens was observed with anti-H-2K plus anti-Ia reactivity. Reactions were observed with all three types of anti-Ia sera and rat lymphocytes. The low level of lysis obtained with all but A.TL anti-A.TH was consistent with the relatively low B-cell percentage in rat lymph node populations. In order to confirm these low percentage lytic reactions as well as the specificity of a select number of anti-Ia sera for B cells, T cells were depleted from lymph node suspensions by treatment with anti-Pta + C' and the surviving B cells tested with anit-Ia sera + C'. The results of these tests are included in Table 3. All tested anti-Ia sera demonstrated enhanced reactivity on enriched B cells from all tested rat strains with the exception of A.TL anti-A.TH. A.TL anti-A.TH lysed high percentages of both B cells and untreated lymph node cells of all tested strains except BN in which apparently T cells were predominantly lysed. It would appear, therefore, that A.TL anti-A.TH has both anti-B- and anti-T-cell activity when tested on rat lymph node cells, a point which will be discussed later. The results of these direct complement-dependent cytotoxicity tests using mouse alloantisera with restricted specificity for I-A and I-E subregion products demonstrate that mouse Ia alloantisera cross-react with rat B cells. Our results suggest that all tested rat strains express both I-A and I-E subregion gene products.

Table 3. Direct Complement-Dependent Cytotoxicity of Mouse Anti-Ia Sera With Rat Lymphocytes

Antiserum	Specificity	Fischer		BN.WF		BN		BN.DA		August		Buffalo		INR
		Whole	B											
A.TH α A.TL	1,2,3,7	> 1/160 (56%)	> 1/160 (95%)	> 1/160 (44%)	> 1/160 (90%)	> 1/160 (54%)	> 1/160 (80%)	> 1/160 (49%)	> 1/160 (95%)	> 1/160 (41%)	> 1/160 (85%)	> 1/160 (38%)	> 1/160 (76%)	> 1/80 (51%)
(B10 x A.TH)αA.TL	1,2,7	> 1/80 (59%)	NT	> 1/80 (47%)	NT	> 1/80 (53%)	NT	> 1/80 (65%)	NT	> 1/80 (70%)	NT	1/10 (42%)	NT	> 1/80 (51%)
A.TL α A.TH	4,5,9,12[a]	> 1/160 (100%)	> 1/160 (100%)	1/40 (71%)	1/80 (74%)	1/160 (70%)	1/10 (34%)	1/80 (60%)	1/40 (80%)	1/80 (79%)	1/160 (73%)	1/80 (80%)	1/160 (88%)	1/20 (42%)
(A.TL x B10.A(5R)αA.TH	4,5,12	1/40 (54%)	1/80 (95%)	1/80 (33%)	1/40 (75%)	1/80 (58%)	1/40 (71%)	1/40 (49%)	1/40 (75%)	1/40 (68%)	1/40 (62%)	1/40 (30%)	1/80 (65%)	1/20 (50%)
(A.TH x B10.HTT)αA.TL	1,2,3	> 1/160 (65%)	> 1/160 (80%)	> 1/160 (37%)	> 1/160 (85%)	> 1/160 (40%)	> 1/160 (85%)	> 1/160 (40%)	> 1/160 (80%)	> 1/160 (37%)	> 1/160 (65%)	> 1/160 (30%)	> 1/160 (76%)	> 1/160 (56%)
(A.BY x B10.HTT)αA.TL	1,2	1/20 (48%)	1/40 (70%)	1/80 (40%)	1/80 (80%)	1/160 (40%)	1/160 (66%)	1/80 (40%)	1/160 (60%)	1/80 (39%)	1/80 (70%)	1/20 (25%)	1/40 (58%)	1/10 (40%)
(A x B10.D2)αB10.A(5R)	8,9,21	> 1/160 (72%)	> 1/160 (85%)	1/40 (41%)	> 1/160 (80%)	1/80 (40%)	> 1/160 (65%)	> 1/160 (42%)	> 1/160 (71%)	> 1/160 (35%)	> 1/160 (80%)	1/80 (35%)	1/80 (85%)	1/160 (45%)
(B10 x HTI)αB10.A(5R)	7,22	1/40 (46%)	1/80 (90%)	> 1/160 (39%)	> 1/160 (85%)	1/40 (37%)	1/80 (80%)	> 1/160 (38%)	> 1/160 (90%)	> 1/160 (43%)	> 1/160 (80%)	> 1/160 (30%)	> 1/160 (63%)	> 1/160 (63%)

[a] A.TL α A.TH also has specificity for Qa-2 and Tla 1, 2, 3.

NT Not tested.

In order to confirm the specificity of these mouse antisera for the respective analogous gene products in rats, absorption analyses and immunoprecipitations were performed.

Absorption Analysis of Mouse Antiserum Demonstrate Several New Serological Specificities on Rat B Cells. Three I subregion-specific alloantisera were selected for absorption analysis: (B10 x HTI)F_1 anti-B10.A(5R) (anti-I-E^k), (A x B10.D2)F_1 anti-A.TL (anti-I-A^k), and (A.TH x B10.HTT)F_1 anti-A.TL. Antisera were diluted 1/10 and 'X' µl absorbed with 'X' x 10^6 spleen cells from each rat strain and positive and negative mouse strains. The absorbed antisera were diluted serially 1/2 → 1/8 (actual dilutions of antisera of 1/20 → 1/160) and tested with rat lymph node B cells.

The results of the absorption of anti-I-E^k serum are presented in Table 4 and Figure 1. The important points of the absorption are 1) the positive mouse cells (B10.A(5R)) absorbed activity for all rat strains, 2) the negative mouse strain cell (B10) did not absorb activity for any rat B cells, and 3) spleen cells from all rat strains cross-absorbed reactivity for target B cells from all other strains. These results confirmed the specificity of the reactivity of the (B10 x HTI)F_1 anti-B10.A(5R) antiserum for rat B cells and demonstrated the existence of specificity RT1.B.12 common to molecules coded for by the gene which appears to be the rat equivalent of the mouse I-E subregion.

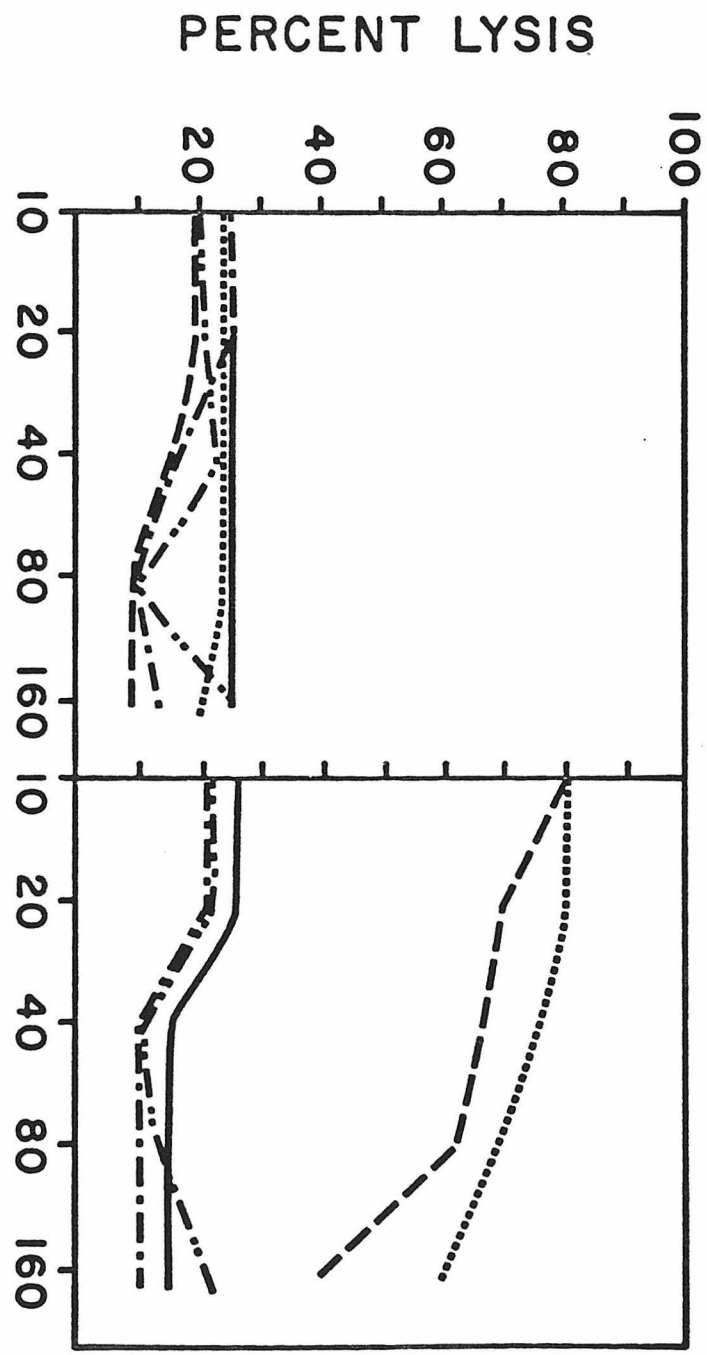
The results of the absorption of (A x B10.D2)F_1 anti-B10.A(5R) (anti-I-A^b) are presented in Table 5. Absorption with mouse spleen cells demonstrated the specificity of the reaction of this antiserum with rat B cells, since B10.A(5R) but not B10.A spleen cells absorbed activity for all rat B cells. Evidence suggesting reactivity of anti-I-A^b with two specificities on rat cells was obtained. As illustrated in Table 5 and in part in Figure 2, reactivity of anti-I-A^b for Fischer and Buffalo B cells could only be completely absorbed with Fischer and Buffalo spleen

Table 4. Absorption of (B10 x HTI) Anti-B10.A(5R) (Anti-I-E^k) With Mouse and Rat Spleen Cells

Absorbing cells	Activity (50% titera) after absorption when tested on B cells from:					
	Fischer	BN.WF	BN	BN.DA	August	Buffalo
Fischer	-	-(1/10)	-	-	-	-
BN.WF	-	-	-	-	-	-
Lew.BN	-(1/20)	-(1/10)	-	-(1/10)	-(1/20)	-
BN.DA	-	-	-	-	-	-
August	-	-	-	-	-	-
Buffalo	-	-(1/10)	-	-	-	-
MNR	-	-	-	-	-	-
B10.A(5R)	-(1/10)	-	-	-	-	-(1/20)
B10	+(>1/160)	+(>1/160)	+(1/80)	+(>1/160)	+(>1/160)	+(>1/160)

Last dilution resulting in 50% of maximum lysis obtained with unabsorbed antiserum.

Fig. 1. Lysis in complement-dependent cytotoxicity with (B10 x HTI) anti-B10.A(5R) (anti-I-E^k) and BN B cells after absorption with the following cells: Left: _____, Fischer; __..__, WF; ....., BN; __.__, BN.DA; __ __ __, August. Right: _____ Buffalo; __..__ MNR; __.__, B10.A(5R); __ __ __, B10; ..... Unabsorbed.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160
1/ANTISERUM DILUTION

Figure 1

Table 5. Absorption of (A x B10.D2) Anti-B10.A(5R) (Anti-I-A^b) With Mouse and Rat Spleen Cells

Absorbing cells	Activity (50% titer) after absorption when tested on B cells from:					
	Fischer	BN.WF	BN	BN.DA	August	Buffalo
Fischer	-	-	-	-	-	-
BN.WF	+(1/80)	-	-	-	-	+(>1/160)
Lew.BN	+(1/80)	+(1/40)	-	-	-	+(>1/160)
BN.DA	+(1/80)	+(1/40)	-	-	-(1/20)	+(>1/160)
August	+(1/80)	-(1/10)	-	-	-	+(1/160)
Buffalo	-	-	-	-	-	-
MNR	+(1/80)	-	-	-	-	+(>1/160)
B10.A(5R)	-	-	-	-	-	-
B10.A	+(>1/160)	+(1/160)	+(1/40)	+(1/80)	+(>1/160)	+(>1/160)

See Table 4.

Fig. 2. Lysis ..... with (A x B10.D2) anti-B10.A(5R) (anti-I-A^b) and Buffalo B cells: Left: _____, Fischer; __._, BN.WF; ....., BN; __.._, BN.DA, __ __ __, August. Right: _____, Buffalo; __.._, MNR; ....., B10.A(5R); __ __ __, B10.A.

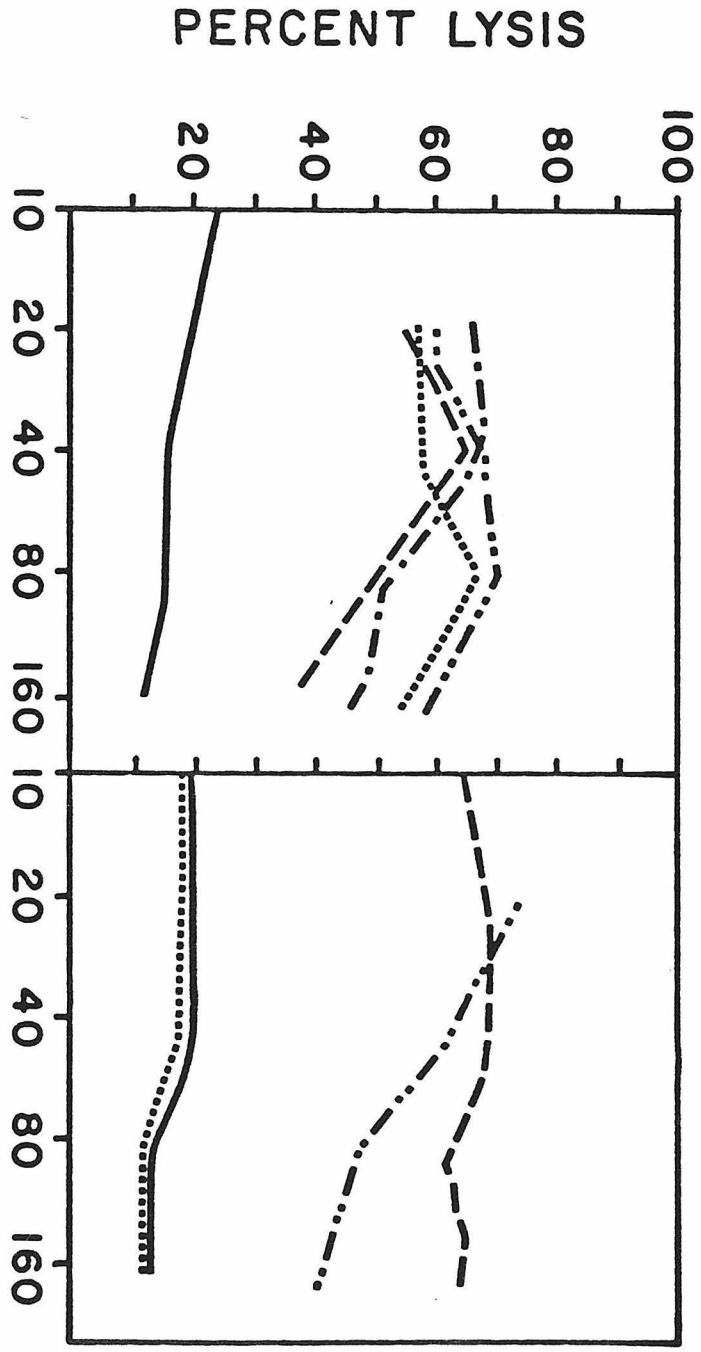
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Figure 2

cells. However, reactivity with BN.WF, BN, BN.DA and August was absorbed with cells from all strains. Therefore, a single specificity B.13 was shared by all six tested haplotypes. Specificity B.14 is expressed by the $\underline{RT1}^{l}$ and $\underline{RT1}^{b}$ haplotypes.

The results of the absorption of (A.TH x B10.HTT)F_1 anti-A.TL are presented in Table 6. The pattern of absorption is considerably more complex than that obtained with anti-I-E^k and anti-I-A^b. The ability of B10.A cells to absorb reactivity of this antiserum for rat B cells and the failure of B10.A(9R) cells to absorb activity confirmed the antigenic specificity of the reaction of anti-I-A^k with rat B cells. Absorption with cells expressing the different RT1 haplotypes demonstrated the presence of no fewer than three new specificities. It should be pointed out that the specificities designated here are minimum numbers of specificities. The first specificity, B.15, was expressed by all tested haplotypes in that all strains absorbed for reactivity on Fischer and BN.DA. This specificity is different than B.13 in that the B.13 specificity is detected by I-A^k anti-I-A^b whereas the B.15 specificity is detected by I-A^s anti-I-A^k. The second specificity detected by anti-I-A^k, B.16, is expressed by the $\underline{RT1}^{w}$, $\underline{RT1}^{n}$, and $\underline{RT1}^{c}$ haplotypes as demonstrated by their ability to remove activity for $\underline{RT1}^{w}$ and $\underline{RT1}^{n}$. Cells of the $\underline{RT1}^{l}$, $\underline{RT1}^{a}$, or $\underline{RT1}^{b}$ haplotype were not able to absorb this reactivity. Figure 3 presents the absorbed antiserum titrations on BN.WF targets. We conclude the $\underline{RT1}^{w}$, $\underline{RT1}^{n}$, and $\underline{RT1}^{c}$ haplotypes share the B.16 specificity. An additional specificity was detected to be expressed only by cells of the $\underline{RT1}^{c}$ haplotype; only August cells could completely absorb activity for August B cells. This specificity has been designated B.17 (Table 7).

Immunoprecipitation of Class II Molecules. Rat class II molecules can be immunoprecipitated using mouse anti-Ia sera (Sporer et al. 1979; Blankenhorn et al. 1980). Profiles of the SDS polyacrylamide disc gels showing the class II molecules isolated from the MNR strain and electrophoresed under reducing and non-reducing conditions are shown in Figure 4. These profiles illustrate two points.

Table 6. Absorption of (A.TH x B10.HTT) Anti-A.TL (Anti-I-A^k) With Mouse and Rat Spleen Cells

Absorbing cells	Activity (50% titer) after absorption when tested on B cells from:				
	Fischer	BN.WF	BN	BN.DA	August
Fischer	-	+(>1/160)	+(>1/160)	-	+(>1/160)
BN.WF	-(1/10)	-	-	-(1/10)	+(1/40)
BN	-(1/10)	-(1/10)	-	-(1/10)	+(1/40)
BN.DA	-	+(>1/160)	+(1/80)	-	+(>1/160)
August	-	-	-	-	-
Buffalo	-	+(1/80)	+(1/40)	-	+(1/80)
B10.A	-	-	-	-	-
B10.HTT	+(1/40)	+(1/160)	+(>1/160)	+(1/40)	+(>1/160)

See Table 4.

Fig. 3. Lysis. (A.TH x B10.HTT) anti-A.TL (anti-I-A^k) and BN.WF B cells: Left: _____, Fischer; __.__, BN.WF; ....., BN; __..__, BN.DA; __ __ __, August. Right: __.__, Buffalo, __ __ __ B10.A; _____, B10.HTT; ....., Unabsorbed.

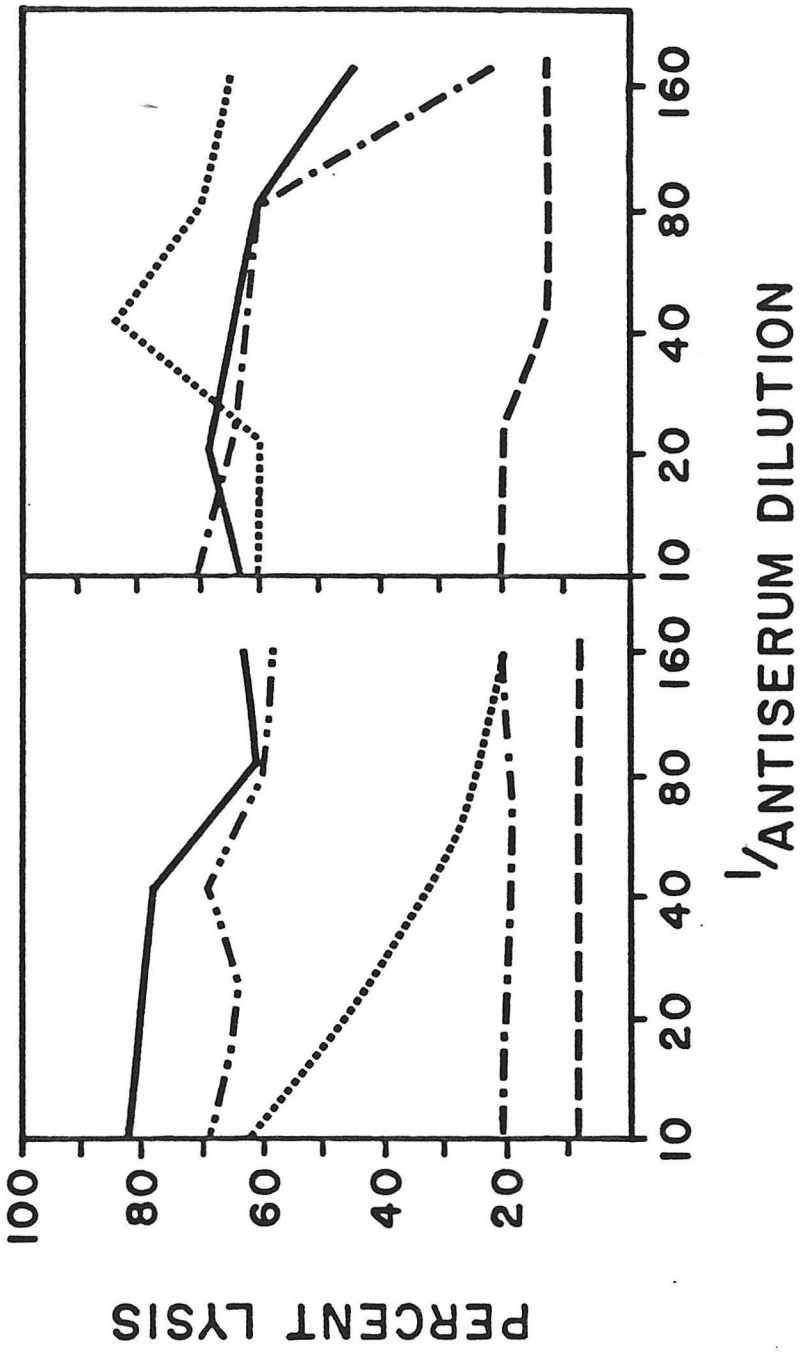
PERCENT LYSIS
100
80
60
40
20
10
20
40
80
160
1/ANTISERUM DILUTION

Figure 3

Table 7. RT1 Distribution of B Specificities Detected by Mouse Anti-Ia Sera

RT1 haplotype	B Specificity					
	12	13	14	15	16	17
RT1^{l}	1	2	3	4	-	-
RT1^{w}	1	2	-	4	5	-
RT1^{n}	1	2	-	4	5	-
RT1^{a}	1	2	-	4	-	-
RT1^{c}	1	2	-	4	5	6
RT1^{b}	1	2	3	4	-	-
RT1^{m}	1	2	-	-	-	-

Fig. 4. Profiles of SDS-polyacrylamide gel electrophoresis of indirect immune precipitates using the monoclonal anti-I-A^k reagent. (---) denotes reducing conditions. (——) denotes nonreducing conditions. Profiles are superimposed for comparative purposes.

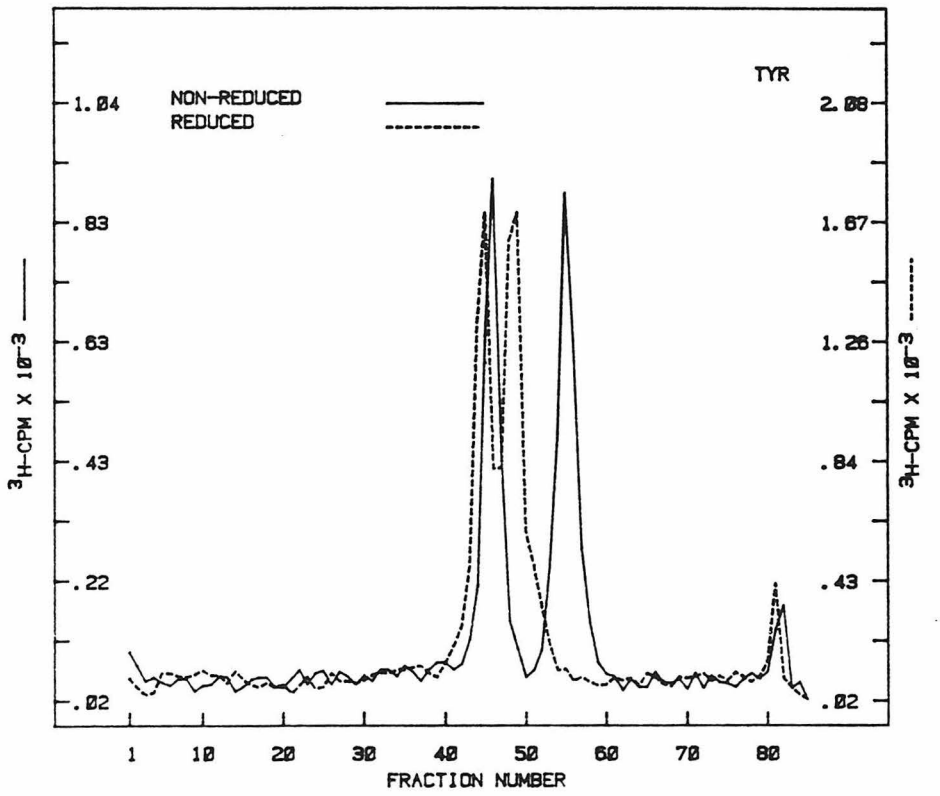
TYR
NON-REDUCED
REDUCED
1.04
.83
.63
.43
.22
.02
2.08
1.67
1.26
.84
.43
.02
^{3}H-CPM X 10^{-3} ——
^{3}H-CPM X 10^{-3} ------
1
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 4

First, two components, α and β, are seen, and these correspond in apparent molecular weight to Ia molecules isolated from mice. Second, the α and β components shift in apparent molecular weight depending on whether they are electrophoresed under reducing conditions (α = 33,000 and β = 31,000) or non-reducing conditions (α = 32,000 and β = 27,000). This is consistent with other recent reports (McMaster and Williams 1979). Because this sort of analysis using disc gels is relatively tedious and inaccurate when comparing large numbers of samples over a period of time, we decided to analyze our immunoprecipitates utilizing SDS slab gels and fluorography. Accordingly, class II molecules from rat lymphocytes expressing the seven common RT1 haplotypes were immunoprecipitated and electrophoresed on polyacrylamide SDS slab gels. The resulting slab gel profiles have been analyzed and the relative intensity of the class II bands are presented in Table 8. The calculated molecular weights of the class II molecules immunoprecipitated by mouse anti-Ia sera are presented in Table 9. As an example of these data, the fluorographs of the gel profiles of the Fischer and MNR strains are presented in Figure 5. Figure 5 and Tables 8 and 9 illustrate several important points. (1) As mentioned above, the apparent molecular weights of the rat class II molecules roughly correspond to their mouse counterparts. (2) In contrast to the mouse, all tested strains express I-E molecules confirming the serological observations described above. (3) The pattern of reactivity of class II antigens of these strains with anti-I-A sera differs from strain to strain. For example, the (A x B10.D2)F_1 anti-B10.A(5R) (anti-I-A^b) antiserum reacts strongly with the Fischer rat strain and very weakly with the MNR strain (Table 9, Figures 4 and 5). (4) The molecular weights of the α and β subunits of the I-A and I-E molecules differ depending on the strain of rat. For example, it is particularly striking that the α and β subunits isolated from MNR using anti-I-A sera are very close in molecular weight (α = 33,000 and β = 31,000) whereas the α and β subunits isolated using the same sera from the Fischer

Table 8. Relative Reactivity of Mouse Anti-Ia Sera With Rat Class II Antigens Assayed by Immunoprecipitation

Sera	MHC Regions recognized	BN	Fischer	August	MNR	DA	Buffalo	WF
$(A.TH \times HTT)F_1$ $\alpha A.TL$	$A^kB^kJ^k$	++	++	+++	++++	+++	++	+++
αA^k monoclonal	A^k	++++	+	++++	++++	++	++	+++
A.TL α A.TH	I^s	-	+	-	-	-	±	±
$(A \times B10.D2)F_1$ B10.A(5R)	$K^bA^bB^b$	+	+++	+	+	+	+	+
$(B10 \times HTI)F_1$ α B10.A(5R)	E^dC^k	+++	++++	+++	+++	+++	+++	+++
NMS	-	-	-	-	-	-	-	-

Table 9. Apparent Molecular Weights of the α and β Subunits Precipitated with Anit-I-A and Anti-I-E Sera

		BN	Fischer	August	MNR	BN.DA	Buffalo	BN.WF
Anti-I-A	α	34	34	33	33	31.5	34	32.5
	β	30	30	31	31	29	29	28
Anti-I-E	α	34.5	33	34	34	33	35	34
	β	28.5	28	28	28	28	30	28

Fig. 5. Fluorographs of SDS-polyacrylamide slab gels electrophoresed under reducing condition. a) Fischer rat strain extracts immunoprecipitated with: Lane 1, normal mouse serum; Lane 2, A.TL anti-A.TH (anti-I^S) but since no E^S product has been isolated this is denoted A^S in the figure; Lane 3, (A x B10.D2)F_1 anti-B10.A(5R) denoted A^b; Lane 4, (A.TH x B10.HTT)F_1 anti-A.TL denoted A^k; Lane 5, monoclonal anti-A^k (10-2.16 monoclonal) denoted A^k; Lane 6, (B10 x HTI)F_1 anti-B10.A(5R) denoted E_k. b) MNR rat strain extracts immunoprecipitated as above except Lanes 4 and 5 are reversed. Molecular weight standards were on the other lanes (not shown). Gels were overloaded to insure detections of weak reactions. The Fischer fluorograph was exposed for 4 days and the MNR for 2 days at -70°C.

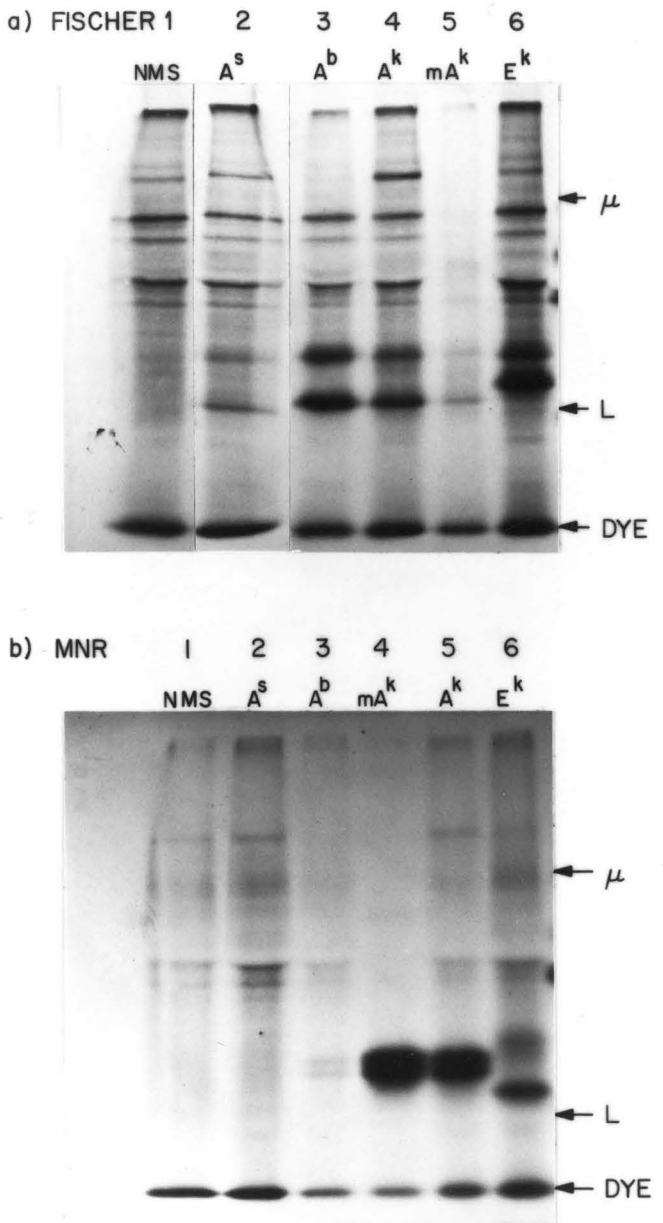
a) FISCHER 1
2
3
4
5
6
NMS
A^s
A^b
A^k
mA^k
E^k
μ
L
DYE
b) MNR
1
2
3
4
5
6
NMS
A^s
A^b
mA^k
A^k
E^k
μ
L
DYE

Figure 5

strain are considerably different (α = 34,000 and β = 31,000). The converse is observed for the class II molecules isolated using mouse anti-I-E serum. Many of the other strains exhibit similar molecular weight variations (Table 9).

Discussion

The Murine Anti-Ia Sera Reactions with Rat Lymphocytes Suggest the Class II Products of Mice and Rats Are Similar. A panel of anti-Ia antisera were tested in complement-dependent cytotoxicity tests with lymphocytes from seven rat strains expressing different RT1 haplotypes. Extensive cross-reactions were observed on rat cells with mouse anti-Ia sera. Our results are consistent with earlier reported results (Shinohara et al. 1978). Tests on T-cell-depleted (B cell) populations with whole lymph node populations demonstrated that most antisera reacted with B cells alone with the exception of A.TL anti-A.TH which had additional reactivity for T cells in most strains. Absorption analyses of some of these antisera demonstrated several points. First, the reactivity of the anti-Ia sera could be absorbed completely with mouse cells expressing the appropriate H-2 haplotype, and was unaffected by mouse cells expressing the inappropriate H-2 haplotype. Second, all rat B cells react with anti-I-E^k serum and share a common specificity. Third, reactions of anti-I-A^b and anti-I-A^k sera with rat B cells defined five additional specificities, two of which were common to all haplotypes. The specificities detected on rat B cells with mouse alloantisera are presented in Table 7. The B (class II) specificities described in this communication are consecutively numbered and numerically follow those defined by Götze et al. (1978) (also D. Götze, personal communication).

Additional Antigens Are Detected by Mouse Anti-Ia Sera. At least two of the antisera contain antibodies to antigens in addition to their anti-Ia antibodies. For example, one of the antisera (A x B10.D2)F_1 anti-B10.A(5R) contains antibodies

to the H-2K^b molecules (a class I molecule). However, no reactivity specific for class I antigens was observed, as it never reacted with 100% of the lymphocytes. In fact, a panel of NIH antisera-specific for H-2K and H-2D antigens were tested for reactivity by direct cytotoxic test with rat lymphocytes; no class I reactivity was observed (P. Wettstein, unpublished observations). The observation that A.TL anti-A.TH kills a large percentage of rat lymphocytes (60-100%) strongly suggests that this antiserum reacts with both T and B cells. This is not surprising since this antiserum reacts with Tla antigens expressed on thymocytes (L. Flaherty, unpublished observations), and reacts with an additional antigen(s) expressed on peripheral lymphocytes (Flaherty et al. 1977).

Immunoprecipitation of Rat Ia Molecules Directly Demonstrate Polymorphism. Immunoprecipitation analyses with radiolabeled rat cell surface molecules and mouse anti-Ia sera indicate that the sera react with the Ia equivalents in the rat. Molecules composed of α and β subunits with molecular weights of approximately 33,000 and 29,000, respectively, were precipitated with anti-Ia sera and Staph A. Of particular interest was the observation that the α and β subunits of rat Ia were polymorphic and their molecular weight apparently varied according to the RT1 haplotype.

The Serological and Biochemical Analyses are Correlated in Several Ways. The immunoprecipitation studies and the serological studies correlated in three ways. First, both the immunoprecipitation and serological analyses indicate that the mouse anti-Ia sera are reacting with rat Ia equivalent. Second, relatively higher titered antisera generally precipitated rat Ia more effectively than low titered antisera, e.g., (A.TH x B10.HTT)F_1 anti-A.TL vs. A.TL anti-A.TH. However, it should be noted that this antiserum, A.TL anti-A.TH, reacts well in an immunoprecipitation assay with mouse cells (Frelinger et al. 1978). Third, the only strains strongly reactive with the anti-I-A^k monoclonal antibody were strains expressing

the B.16 specificity: BN.WF, BN, August, and MNR (absorption analyses not shown).

The Scarcity of RT1 Recombinants May Be Due to a Limitation in the Detection Method. The characterization of the RT1 system has progressed slowly compared to the characterization of the MHCs of mice, guinea pigs and humans. This progress has been hindered primarily by the lack of knowledge of the genetic organization of the RT1 complex due to the low number of documented intra-RT1 recombinants. Although recombinations have been identified which occurred between the two classes of genes, no laboratory recombinants have been reported which occurred within either class. The inability to detect recombinations in these positions may be due to either a relatively low frequency of recombination between these loci or limitations in the typing procedures previously employed. There is little information on the importance of either alternative. What is clear is that the identification of these recombinants requires the detection of products of genes on both sides of the recombinations. One problem is that rat alloantisera do not always contain activity for all expected RT1 antigens. An analogous situation has been observed frequently in mouse alloantisera. Mixed lymphocyte culture (MLC) typing suffers from the same limitations. This is exemplified by the lack of proliferation in MLC combinations of MNR and August cells (Cramer et al. 1977) which apparently have a common class II allele while differing at the second class II gene (Sporer et al. 1979). The combination of these limitations and perhaps the order of the genes in RT1 may severely hinder the detection of certain types of intra-RT1 recombinations. To overcome these obstacles and to improve the efficiency of serologically identifying intra-RT1 recombiantions we believed it was important to first select serological reagents with defined specificity. The reports of Shinohara et al. (1977) and Blankenhorn et al. (1980) suggested to us that cross-reactive mouse anti-Ia sera might be used to detect intra-RT1 recombinants. It was with this goal in mind that we began the analyses of these cross-reactive anti-Ia sera.

The Mouse Anti-Ia Cross-Reaction with Rat Class II Molecules Will Be Very Useful in Analyzing the RT1 Complex. Although our studies demonstrated polymorphic cross-reactions between mouse anti-Ia sera and rat class II antigens, we do not believe these antisera will be directly useful in recombinant screens. However, the polymorphism in the molecular weights of the α and β subunits of rat class II molecules precipitated with mouse alloantisera suggested that mouse anti-Ia sera might be useful in the characterization of rat alloantisera for their class II reactivity. For example, we hypothesize that in rat strains where the α and β subunits of the two RT1 class II gene products differ in their molecular weights it should be possible to determine the specificity of rat alloantisera by comparing the molecular weights of the α and β subunits precipitated with rat alloantisera and the mouse anti-Ia sera. This approach has proven fruitful in the determination of the origins of the class II genes of August and MNR. It has been demonstrated by Sporer et al. (1979) and Götze et al. (1979) that $\underline{RT1}^c$ and $\underline{RT1}^m$ have a common class II gene but differ at a second class II gene. The α and β subunits of the I-A and I-E equivalents of $\underline{RT1}^c$ and $\underline{RT1}^m$ haplotypes differ in their molecular weights. Immunoprecipitation analysis of rat alloantisera specific for $\underline{RT1}^c$ and $\underline{RT1}^m$ class II antigens demonstrated that anti-MNR sera cross-reactive with August class II antigens precipitate α and β molecules with characteristics of molecules precipitated with anti-I-A^k.

Conversely, August anti-MNR and MNR anti-August sera precipitate molecules from their respective strains with characteristics of molecules precipitated by anti-I-E^k serum. We are continuing these characterizations of rat alloantisera in order to reveal the spectrum of their reactivity.

Through the use of restricted mouse anti-Ia sera in characterizing rat antiserum reactivity, we expect to be able to select reagents which will be useful in (1) the identification of intra-$\underline{RT1}$ recombinants, (2) the determination of the

relative role of rat class II antigens as stimulator antigens in MLC, and (3) the analysis of the participation of rat class II molecules in regulating antigen presentation, macrophage:T cell, and T:B cell interactions in the humoral response.

References

Blankenhorn, E. P., Cecka, J. M., Frelinger, J. G., Götze, D., and Hood, L.: Structure of Ia antigens in rats. Eur. J. Immunol. 10:145-151, 1980.

Blankenhorn, E. P., Cecka, J. M., Götze, D., and Hood, L.: Partial N-terminal amino acid sequence of rat transplantation antigens. Nature 274:90-92, 1978

Bogden, A. E., and Aptekman, P. M.: The "R-1" factor: A histocompatibility antigen in the rat. Cancer Res. 20:1272-1282, 1960

Butcher, G. W., and Howard, J. C.: Pta (alius Ag-F): An alloantigenic system on rat peripheral T cells. Rat Newsletter 1:12, 1977

Cecka, J. M., Blankenhorn, E. P., Götze, D., and Hood, L.: Structure of Ia antigens from rats: I. Microsequence analyses of Ia antigens from three strains of rats. Eur. J. Immunol.(in press) 1980

Cramer, D. V., Davis, B. K., Shonnard, J. W., and Gill, T. J.: The graft-versus-host reactivity in Ag-B/MLR disparate strains of rats. Transplantation 23:498-503, 1977

Cullen, S. E., and Schwartz, B. D.: An improved method for the isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphlococci. J. Immunol. 117:136-142, 1976

Davidson, W. F., and Parish, C. R.: A procedure for removing red cells and dead cells from lymphoid cell suspensions. J. Immunol. Meth. 7:291, 1975

Flaherty, L., Stanton, T. H., and Boyse, E. A.: Contamination of Ia antisera with antibodies related to the Tla-region. Immunogenetics 4:101-103, 1977

Frelinger, J. A., Niederhuber, J. E., David, C. S., and Shreffler, D. C.: Evidence for the expression of Ia (H-2 associated) antigens or thymus-derived lymphocytes. J. Exp. Med. 140:1273, 1974

Frelinger, J. G., Wettstein, P. J., Frelinger, J. A., and Hood, L.: Epidermal Ia molecules from the I-A and I-EC subregions of the mouse H-2 complex. Immunogenetics 6:125-135, 1978

Götze, D.: The major histocompatibility complex of the rat, RT1. I. The serological characterization of the MNR haplotype ($RT1^m$) in regard to the cross-reacting haplotypes $RT1^a$, $RT1^c$, and $RT1^b$. Immunogenetics 7:491-506, 1978

Hämmerling, G. J., Mauve, G., Goldberg, E., and McDevitt, H. O.: Ia on spermatozoa, macrophages and epidermal cells. Immunogenetics 1:428, 1975

Klein, J.: Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag, New York, 1975

Kren, V., Vesely, P., Frenzyl, B., and Stark, O.: Inhibition of the uniting syndrome in rats. Folia Biol. (Praha) 6:333-341, 1960

Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 19

Laskey, R. A., and Mills, A. D.: Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341, 1975

McMaster, W. R., and Williams, A. F.: Monoclonal antibodies to Ia antigens from rat thymus: Cross reactions with mouse and human and use in purification of rat Ia glycoproteins. Immunological Rev. 47:117-137, 1979

Niederhuber, J. E., Frelinger, J. A., Dugan, E., Coutiuho, A., and Shreffler, D. C.: Effects of anti-Ia serum on mitogenic responses. J. Immunol. 115:1672-1676, 1975

Palm, J.: Current status of blood groups in rats. Ann. N.Y. Acad. Sci. 97:57-68 1962

Sachs, D. H., and Cone, J. L.: Ia-associated murine alloantigens: Demonstration of multiple Ia specificities in H-2 alloantisera after selective absorption. J. Immunol. 114:165, 1975

Sachs, D. H., Humphrey, G. W., and Lunney, J. K.: Sharing of Ia antigens between species. J. Exp. Med. 146:381-393, 1977

Shinohara, N., Lunney, J. K., and Sachs, D. H.: Sharing of Ia antigens between species. J. Immunol. 121:637-640, 1978

Silvers, W. K., Wilson, D. B., and Palm, J.: Mixed leukocyte reactions and histocompatibility in rats. Science 155:703-704, 1967

Sporer, R., Manson, L. A., and Götze, D.: A biochemical analysis of the membrane-associated gene products of major histocompatibility complex of the rat. J. Immunol. 122:2162-2165, 1979

Chapter VII

Appendix

Appendix

Analyses of RT1 Products using Two-Dimensional Polyacrylamide Gels

The data presented in this appendix represents an extension of the one-dimensional gel analyses presented in Chapter VI. Two-dimensional gel analyses were carried out as described previously (1) with minor modifications as outlined in the Legend to Figure 1. Several conclusions can be drawn from these data.

1) The two-dimensional gels nicely separate the Class I, the Class II "A" and the Class II "E" molecules from one another (Figure 1).

2) The Class I molecules immunoprecipitated with heterologous rabbit anti-β_2-microglobulin antiserum are polymorphic (Figure 2).

3) The Class II molecules immunoprecipitated with murine anti-I-A sera are polymorphic (Figure 3).

4) The Class II molecules immunoprecipitated with murine anti-I-E sera are polymorphic (Figure 4) and distinct from the Class II "A" molecules isolated using the anti-I-A sera (Figure 3 and Chapter V).

5) The Class II molecules of the MNR and the August strain of rat are identical by this technique (Figures 3 and 4), while the Class I molecules are quite different (Figure 2).

6) A relatively invariant basic polypeptide is present in all the immunoprecipitations using anti-Ia sera. This polypeptide probably is the rat counterpart of the constant or invariant polypeptide previously reported for mice (2).

Figure 1. Fluorographs of two-dimensional gels of RT1 products. a) Fischer Class I molecules isolated using a rabbit anti-β_2-microglobulin antiserum. b) Fischer Class II "A" molecules isolated using (A.THxB10.HTT)F_1 anti-A.TL (anti-I-A^k). c) Fischer Class II "E" molecules isolated using (B10xHTI)F_1 anti-B10.A(5R) (anti-I-E^k). All samples were loaded on the acidic end of the gel. The sample in a) was focused for 10.5 hours while the samples in b) and c) were focused for 9.5 hours.

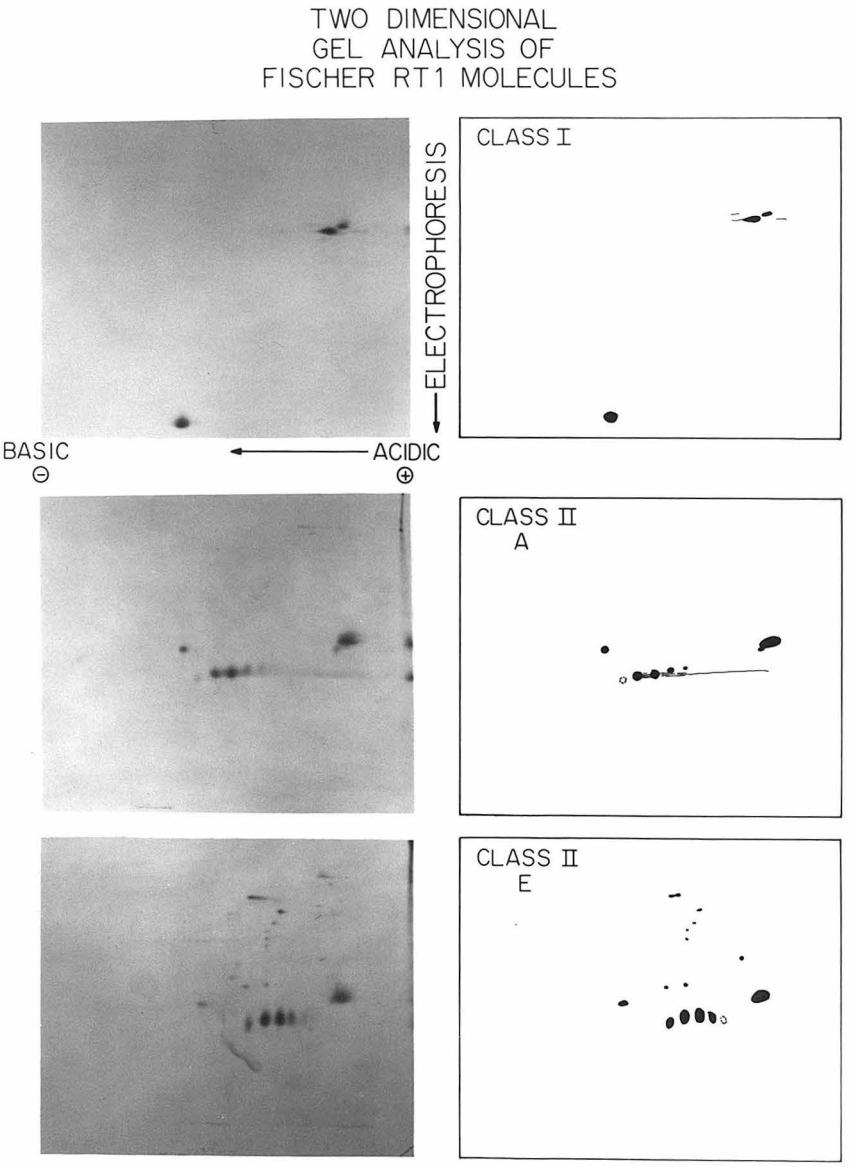
TWO DIMENSIONAL
GEL ANALYSIS OF
FISCHER RT1 MOLECULES
ELECTROPHORESIS
BASIC
⊖
ACIDIC
⊕
CLASS I
CLASS II
A
CLASS II
E

Figure 1

Figure 2. Fluorographs of RT1 Class I molecules isolated using rabbit anti-β_2-microglobulin. Only the molecular weight range about 45,000 is shown. Rat strains are as labeled in the photograph. The position where the standard proteins ribonuclease A, ovalbumin, and hemoglobin focused is shown at the bottom of the photo.

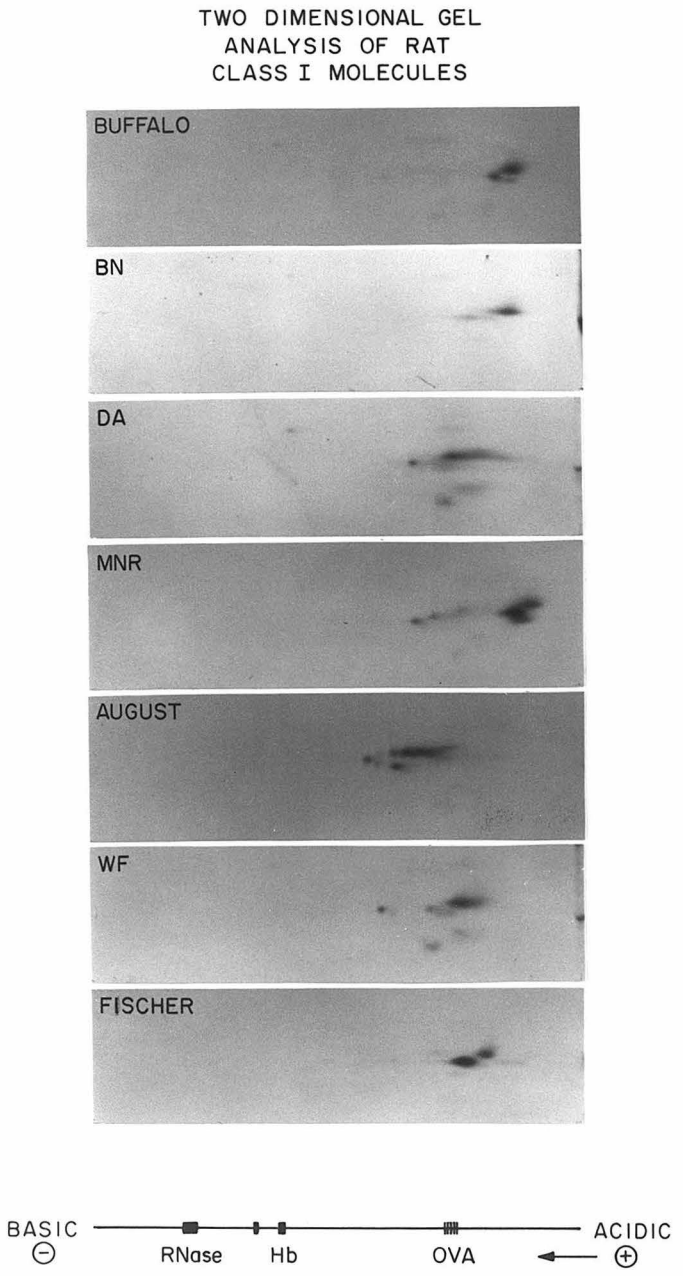
TWO DIMENSIONAL GEL
ANALYSIS OF RAT
CLASS I MOLECULES
BUFFALO
BN
DA
MNR
AUGUST
WF
FISCHER
BASIC
⊖
RNase
Hb
OVA
ACIDIC
⊕

Figure 2

Figure 3. Fluorographs of RT1 Class II molecules isolated using mouse anti-I-A sera. Only the molecular weight range 25,000 to 40,000 is shown. Rat strains are as labeled in the photograph. The position where the standard proteins ribonuclease A, ovalbumin, and hemoglobin focused is shown at the bottom of the photo.

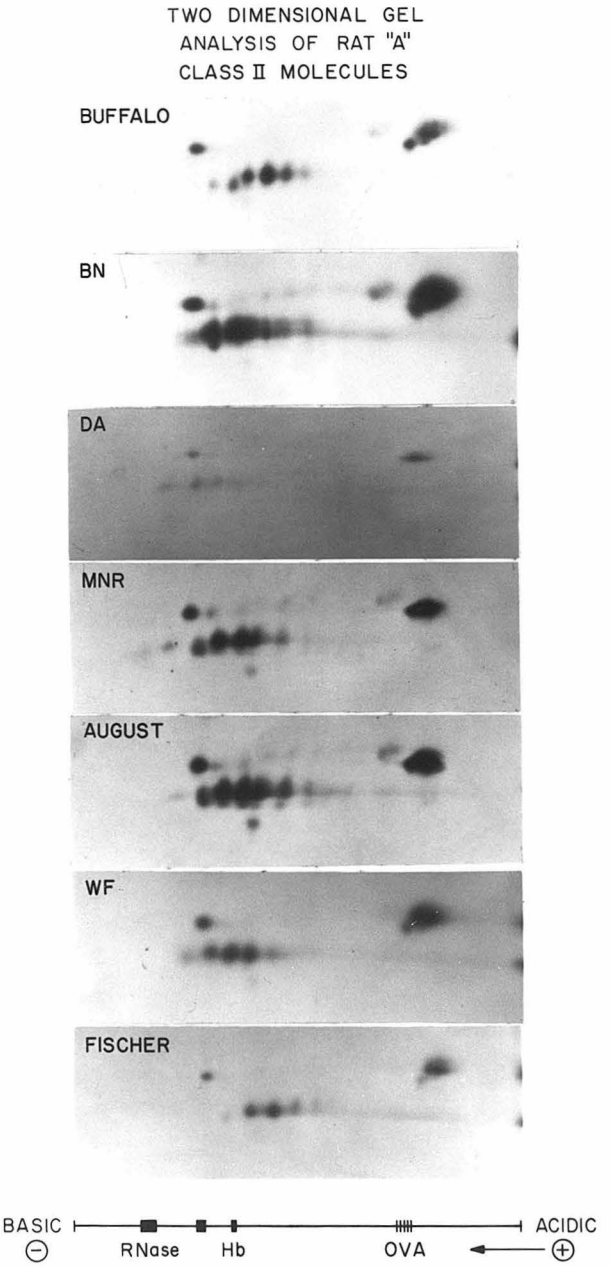
TWO DIMENSIONAL GEL
ANALYSIS OF RAT "A"
CLASS II MOLECULES
BUFFALO
BN
DA
MNR
AUGUST
WF
FISCHER
BASIC
⊖
RNase
Hb
OVA
ACIDIC
⊕

Figure 3

Figure 4. Fluorographs of RT1 Class II molecules isolated using mouse anti-I-E sera. Only the molecular weight range 25,000 to 40,000 is shown. Rat strains are as labeled in the photograph. The position where the standard proteins ribonuclease A, ovalbumin, and hemoglobin focused is shown at the bottom of the photo.

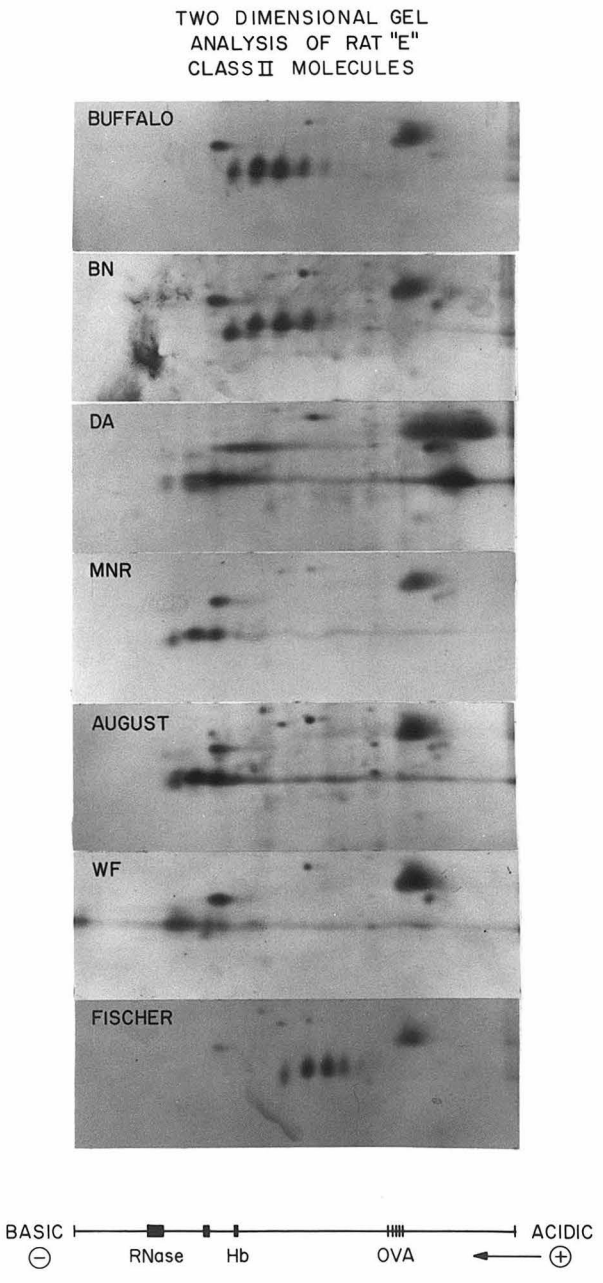
TWO DIMENSIONAL GEL
ANALYSIS OF RAT "E"
CLASS II MOLECULES
BUFFALO
BN
DA
MNR
AUGUST
WF
FISCHER
BASIC
ACIDIC
RNase
Hb
OVA

Figure 4

References

1. O'Farrell, P. H. J. Biol. Chem. **250:** 4007 (1975).

2. Jones, P. P., Murphy, D. B., Hewgill, D., and McDevitt, H. O. Molecular Immunol. **16:** 51 (1979).