IMMUNOGENETIC STUDIES OF THE MOUSE AND THE RAT

Thesis by

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To Tim

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ABSTRACT

This thesis includes immunogenetic investigations of three systems in mice and rats. The first part deals with theta (Thy-1) antigens in mice. Results and conclusions are presented from three separate experiments: 1) Crosses were made between inbred mice, and electrophoretic analysis of serum transferrin and kidney malic enzyme, along with serological detection of Thy-1, aligned the three loci in the following order on chromosome 9: Trf --- Mod-1 --- Thy-1. This work led to a revision of the previously-accepted map of chromosome 9 in the mouse. 2) Sublines of inbred AKR mice, which differ in susceptibility to leukemias, were found to differ from one another by two of these same chromosome 9 genetic markers (Mod-1 and Thy-1). 3) Two alleles of Thy-1 were found to be segregating in one wild mouse colony (from the Southern California area) which has an unusually high incidence of lymphomas. Only one allelic product was detected in another wild mouse colony, which is lymphoma-resistant. However, the inheritance of either allele of Thy-1 could not be associated with an individual predisposition to lymphoma.

The second part of the thesis deals with rat lymphoid cell antigens. In rats, the major histocompatibility complex is called H-1. A study of the membrane-associated glycoprotein products of this genetic region was conducted, employing a number of biochemical techniques, including electrophoretic separation on one-dimensional and two-dimensional gels, and microsequence analysis. The study showed that the major transplantation antigens in rats are closely similar to one another, and homologous to those of other species. The results demonstrate that there may be two transplantation antigens encoded by each of three H-1 haplotypes.

The alloantigens associated with the H-1 linked immune response region in rats (Ia antigens) were also studied, and evidence is presented for the existence of two separate Ia antigens in each rat haplotype studied.

An immunogenetic analysis of the levels of a mouse carcino-embryonic antigen known as alpha fetoprotein (AFP) is contained in the last part of this thesis. An extremely sensitive radioimmunoassay was developed to detect levels of AFP in inbred strains and genetic crosses of mice which produce high or low adult concentrations of this fetal protein. The results from this study indicate no genetic association between the structural gene for albumin and the factor(s) regulating the level of serum AFP. There is also evidence that the high AFP level seen in some of the backcross progeny of the particular cross investigated is not determined by a single recessive gene.

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INTRODUCTION

The field of immunogenetics covers an increasingly wide variety of subjects. The application of immunological techniques to study genetic phenomena and the use of genetically controlled experiments to derive a greater understanding of the functions of the immune system are equally within the scientific territory of an immunogeneticist. The topics covered by this thesis are all loosely linked under the heading "Immunogenetic Studies of the Mouse and the Rat," and the thread of their relatedness is a good example of the power of immunogenetic analysis.

The first part of this thesis summarizes my work on a thymus antigen known in the mouse as theta (or, more recently, Thy-1). This is an antigen with two known alleles, found on thymocytes and thymus-derived lymphocytes, as well as on brain tissue, in the mouse (1). The two alternative forms of the antigen depend on a pair of codominant alleles which are completely penetrant. The immunological distinction between the two allelic forms is very clear and the genotype of a given mouse is easily assessed with a simple serological technique. The Thy-1 locus thus provides the immunogeneticist with a good system to study, and the first three chapters of the thesis are about theta. The work I have included describes three aspects of the Thy-1 antigen system: the mapping of the gene for Thy-1 on the 9th chromosome of the mouse, the variation of the Thy-1 antigen among substrains of AKR inbred mice, and the distribution of the two Thy-1 alleles in populations of wild mice from the Southern California area.

I have placed my work on the rat major histocompatibility complex (MHC) second among the three topics. The MHC is a large genetic region in vertebrates which controls a variety of immunological functions. The rat MHC is called AgB

or H-1. Only two regions within the AgB complex have been described - one contains the gene(s) coding the major transplantation antigens, and the other is a genetic region controlling the rat immune response to a number of antigens. It is my intention to study the proteins isolated from rats using specific antisera directed to the AgB complex, and to draw conclusions about the genetic organization of this complicated system from an analysis of the number and nature of the products it encodes. This is inherently different from the theta antigen work in two ways:

1) The genetic control of the AgB complex is probably more intricate than the single gene associated with Thy-1. It is likely that the Thy-1 locus contains the structural information for the Thy-1 product. The only regulation implied for the gene is in the kinds of cells, and the developmental times, in which it comes to expression. The number of genes within AgB is not known. The existence of multiple, duplicated, and divergent genes in the homologous region of the mouse (2) leads one to suspect that the rat may have the same complexity of organization. The mouse MHC, and perhaps that of the rat, contains several known regulator genes. Furthermore, there have been a number of functions assigned to the rat MHC, whereas Thy-1 enjoys no functional assignments at all.

There exist very few reagents that can dissect and identify separate genes within the rat MHC. While the methods used to distinguish different rat AgB phenotypes are just as simple as the typing involved to distinguish the Thy-1 phenotypes, they may not be so definitive for smaller subregions within this rat complex.

2) Besides the different level of genetic complexity, a second difference between these two systems is that the chemical isolation of AgB molecules is relatively simple when compared to the isolation of the stubbornly hydrophobic

Thy-1 molecules. The intractibility of Thy-1 to chemical purification has been partially overcome and some characterization of the Thy-1a protein has recently been done (3). The rat AgB antigens, on the other hand, have been as manageable as other mammalian MHC antigens when released from lymphoid cells by mild detergent treatment. I have been able to study these MHC molecules which are specifically isolated with rat alloantiserum, and they are the subject of part two of the thesis.

Part two also contains my experiments with an exceptionally useful cross reaction, where I was able to use mouse typing reagents (mouse alloantiserum to specific mouse MHC antigens) to isolate rat AgB molecules. Analysis of these proteins, isolated with both types of reagents, leads to certain conclusions about the genetic apparatus necessary for coding these MHC products. Recombinational events in the rat MHC (at a frequency of less than 0.25% (4)) have provided us with gene assignments no more precise than within one of the two regions already known to exist in the complex. My results, when carefully interpreted, give evidence of greater genetic complexity than has previously been demonstrated.

The last part of this work is dedicated to the quantitative aspect of immunogenetic research. My study of alpha fetoprotein (AFP) in mice has provided virtually no positive results at all, but I believe it is one of the most exciting areas of my research. The powers of immunogenetic methods are no better revealed than in the detection of minute amounts of a fetal protein in the serum of adult mice. AFP is an abundant protein in fetal serum and liver, in a number of mammalian species, that reappears in adults during malignant transformation of the liver. It was my intention to map the structural locus of AFP in mice, but no electrophoretic or immunological variants were detected in any of the inbred mouse

strains that I tested. Therefore, I have included only my work concerning the quantitative regulation of this character, a study which follows the segregation of the AFP-high and AFP-low traits in a controlled mating population of adult mice. This was not entirely successful either, but, nonetheless, it is interesting to hope to discover, for the first time since the genetic mapping of fetal hemoglobin beta chain, the genetic apparatus and strategies that regulate the orderly transition of mammalian fetal proteins to adult homologues during development. My work on this topic is presented in the final chapter.

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II. Location of the Gene for Theta Antigen in the Mouse

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Location of the Gene for Theta Antigen in the Mouse

ELIZABETH P. BLANKENHORN AND TOMMY C. DOUGLAS

THETA (determined by the *Thy-1* locus[25]) is a cell-surface antigen found on mouse thymus-derived lymphocytes and in the brain[17]. Because it is expressed in increasing amounts in developing prenatal thymus and early postnatal brain[15,18], it is a convenient marker for the differentiation state of cells in both tissues. The expression of theta is controlled by an autosomal gene with two known codominant alleles: *Thy-1*^{a} and *Thy-1*^{b}. The mouse strains AKR/J and C3HeB/Fe were used in the reciprocal thymus cell immunizations that produced the original cytotoxic antibody reagents for this antigen[17].

Itakura *et al.*[9] demonstrated linkage between the markers dilute (*d*) and *Thy-1*, placing *Thy-1* in linkage group II. The distance between these two markers was shown to be 16.8 ± 3.8 map units. However, it was not determined on which side of *d* the *Thy-1* gene resided. The purpose of the present study was to define the position of *Thy-1* in linkage group II, which has now been identified as chromosome 9[3].

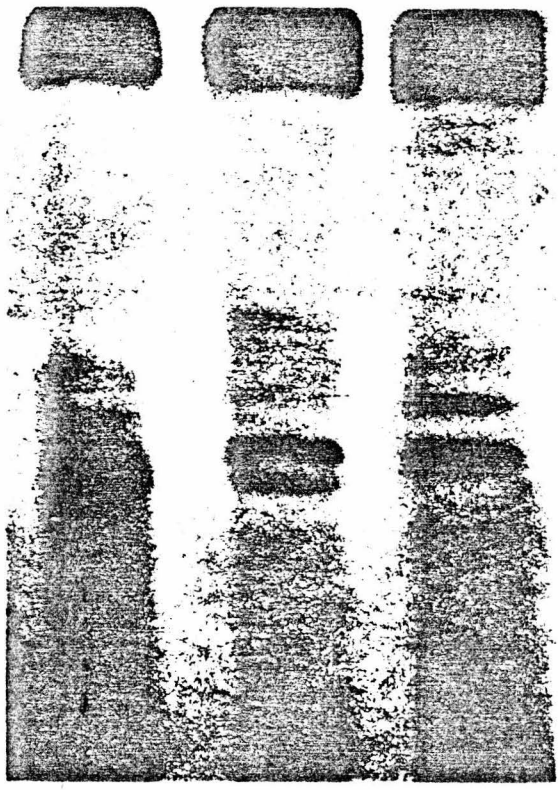
Our experimental design was to measure the recombination between *Thy-1* and *Trf*, the gene controlling an iron-binding serum protein, transferrin. Previously, *Trf* had been placed distal to *d* with respect to the centromere in linkage group II[22]. By observing the frequency of recombination between *Thy-1* and *Trf*, we expected to locate *Thy-1* more precisely. In addition, we followed the segregation at the *H-14* locus, which has not previously been assigned to any linkage group[16,19]. This locus, also known as *Ea-2*, determines a red-blood cell antigen.

During the course of our experiment, Itakura *et al.*[10] reported the linkage of the cytoplasmic malic enzyme locus, *Mod-1*, to *Thy-1* and *d*. We also report data regarding the linkage of this marker, which was segregating in our cross.

Materials and Methods

The F_1 and backcross generations were bred from CBA/J and RF/J mice obtained from The Jackson Laboratory, Bar Harbor, Maine. Samples of blood from the tails of individually numbered mice were typed for both H-14 and transferrin. Red blood cells collected into Gibson's CPD anticoagulant were washed three times in ice-cold saline and tested for H-14 by the PVP hemagglutination method[26]. Serum recovered from depression plates was separated by starch gel electrophoresis and stained with 0.25 percent Coomassie Blue to identify transferrin phenotypes[21,23] (Figure 1). Mice were thymectomized under anesthesia[2] and the thymus cells typed for

FIGURE 1—Patterns obtained by starch gel electrophoresis of sera from mice of transferrin types *b/b* (left), *a/b* (middle), and *a/a* (right). Anode at top of picture.



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theta by direct cytotoxicity using a modification of the method of Amos *et al.*[1]. In later studies, mice were sacrificed and the kidneys and thymus were removed. Kidneys were typed for malic enzyme using the method of Shows and Ruddle[20] with the following modifications: A 12 percent Connaught starch gel was made in 0.008 *M* Tris 0.003 *M* citrate, pH 6.1, and poured into a horizontal Plexiglass template containing 12 upright teeth, which provided sample wells. Kidney preparations were mixed with equal volumes of 2 percent Seaplaque agarose (Marine Colloids, Inc., Rockland, Maine) at 37°C. Samples were added when the gel was cool and run horizontally at 4°C with 0.223 *M* Tris 0.086 *M* citrate (pH 5.6) tank buffer for 5-6 hours at 300 volts (Figure 2).

Results

Reciprocal crosses were made between mice of strains CBA/J and RF/J. The F_1 progeny were then backcrossed to mice of the parental strains. Backcross progeny were typed for theta, transferrin, malic enzyme, and H-14. Mice of strain CBA/J have the genotype *Thy-1*^{b}, *Trf*^{a}, *Mod-1*^{b}, and *H-14*^{z}, while RF/J animals are *Thy-1*^{a}, *Trf*^{b}, *Mod-1*^{a}, and *H-14*^{x}. Because all four of the markers examined in this cross are expressed in a codominant fashion, the genotype of the gamete received from the hybrid parent can be inferred directly. Therefore, in Tables I through III, only those alleles inherited from the hybrid parent are listed. In all cases, the observed phenotypes of the backcross progeny were consistent with the codominant expression of these markers.

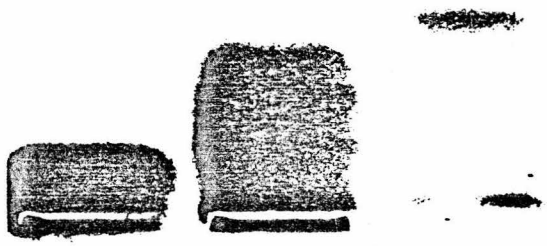


FIGURE 2—Patterns obtained by starch gel electrophoresis of kidney extracts from mice of *Mod-1* types *a/a* (left), *a/b* (middle), and *b/b* (right). Cathode at top of picture.

Table I. Recombination data for *Trf*, *Mod-1*, and *Thy-1*

Hybrid gamete	CR × C*	C × CR	CR × R	R × CR
Parental				
Trf^a *Mod-1*^{b} *Thy-1*^{b}	10	18	21	10
Trf^b *Mod-1*^{a} *Thy-1*^{a}	7	17	19	12
Recombinant, *Trf – Mod-1*				
Trf^a *Mod-1*^{a} *Thy-1*^{a}	0	4	10	1
Trf^b *Mod-1*^{b} *Thy-1*^{b}	4	4	6	1
Recombinant, *Mod-1 – Thy-1*				
Trf^a *Mod-1*^{b} *Thy-1*^{a}	5	12	9	2
Trf^b *Mod-1*^{a} *Thy-1*^{b}	3	4	4	5
Double recombinant				
Trf^a *Mod-1*^{a} *Thy-1*^{b}	0	0	0	0
Trf^b *Mod-1*^{b} *Thy-1*^{a}	0	0	0	0
Total	29	59	69	31
Recombination percentages				
Trf – Mod-1	13.8 ± 6.4	13.6 ± 4.5	23.2 ± 5.1	6.5 ± 4.4
Mod-1 – Thy-1	27.6 ± 8.3	27.1 ± 5.8	18.8 ± 4.7	22.6 ± 7.5
Trf – Thy-1	41.4 ± 9.1	40.7 ± 6.4	42.0 ± 5.9	29.0 ± 8.2

* Strain CBA/J is abbreviated as C, strain RF/J as R, and F_1 hybrids are designated CR

Three-point recombination values for the segregation of *Thy-1*, *Mod-1*, and *Trf* are given in Table I. The lack of recombinants of the reciprocal types Trf^a-$Mod\text{-}1^a$-$Thy\text{-}1^b$ and Trf^b-$Mod\text{-}1^b$-$Thy\text{-}1^a$ indicates that the gene order is *Trf—Mod-1—Thy-1*.

Additional animals were typed for *Trf* and *Thy-1* only. The results are given in Table II, which includes the data from Table I. The pooled estimate of the recombination frequency for crosses in which the hybrid parent was female, with each estimate weighted by the reciprocal of its variance, is 39.6 ± 3.9 percent. The estimate for recombination in the male, calculated in the same way, is 36.3 ± 5.0 percent. The estimates for the two sexes were tested for homogeneity and pooled in the manner described. The overall recombination frequency between *Trf* and *Thy-1* is 38.3 ± 3.1 percent.

Recombination data from progeny typed only for *Trf* and *Mod-1* are shown in Table III, which also includes the data from Table I. Using the same methods as described for the *Trf—Thy-1* data, estimates of 21.1 ± 3.9 percent and 7.7 ± 2.4 percent were obtained for recombination frequencies between *Trf* and *Mod-1* in the female and male, respectively. When tested for homogeneity, these estimates were significantly different ($P < .01$). The arithmetic average of these percentages is 14.4 percent. No significant difference was found, however, between the female and male recombination frequencies for *Mod-1* and *Thy-1*. The pooled estimate for females is 21.0 ± 4.1 percent, and that for males is 25.4 ± 4.6 percent. The overall estimate of the distance between *Mod-1* and *Thy-1* is 22.9 ± 3.1 percent.

Results from 102 backcross animals failed to indicate linkage between *H-14* and either *Trf* or *Thy-1*. The observed recombination frequencies were: *Trf—H-14*, 46.6 ± 4.9 percent; and *Thy-1—H-14*, 51.0 ± 5.0 percent. This finding is in accord with that of Itakura *et al.*[10].

The gene for albinism (*c*, chromosome 7) and the gene for nonagouti (*a*, chromosome 2) could be followed in our cross. Both *H-14* and the markers of the three-point tests on chromosome 9 assorted independently of both coat-color markers.

Table II. Two-point recombination data for *Trf* and *Thy-1*

Hybrid gamete	CR × C*	C × CR	CR × R	R × CR
Parental				
Trf^b $Thy\text{-}1^a$	15	17	30	12
Trf^a $Thy\text{-}1^b$	19	18	29	10
Recombinant				
Trf^b $Thy\text{-}1^b$	15	8	14	6
Trf^a $Thy\text{-}1^a$	10	16	22	3
Total	59	59	95	31
Recombination percentage	42.4 ± 6.4	40.7 ± 6.4	37.9 ± 5.0	29.0 ± 8.2

* Abbreviations same as those used in Table I

Table III. Two-point recombination data for *Trf* and *Mod-1*

Hybrid gamete	CR × C*	C × CR	CR × R	R × CR
Parental				
Trf^b $Mod\text{-}1^a$	14	38	23	17
Trf^a $Mod\text{-}1^b$	18	51	30	12
Recombinant				
Trf^b $Mod\text{-}1^b$	5	4	6	1
Trf^a $Mod\text{-}1^a$	2	4	10	1
Total	39	97	69	31
Recombination percentage	17.9 ± 6.1	8.2 ± 2.8	23.2 ± 5.1	6.5 ± 4.4

* Abbreviations same as those used in Table I

Discussion

Chromosome 9 of the mouse was reviewed several years ago by Green and Lane[7]. In addition to the standard published linkage map[6], an updated version has been compiled by M. C. Green (personal communication). Several markers have been added to this linkage group since 1967, including *Fv-2*[13] (which influences the response to Friend leukemia virus), *Mod-1*, *Thy-1*, and a new hair deficiency (M. C. Green, personal communication). The 14 identified loci in this linkage group code for isoenzymes, surface antigens, morphological and behavioral effects, a serum protein, and a coat color dilution. Translocations have been useful in determining gene order and centromere position for the chromosome. Klein[11] has reported the location of the breakpoint of one translocation [*T*(**9;17**) *138 Ca*]* to be between curly whiskers (*cw*) and short-ear (*se*). Using another translocation, *T*(**9;19**) *163H*, Lyon *et al.*[11] showed that the centromere is located at the end of the linkage group beyond *cw*.

Itakura *et al.* were able to assign *Thy-1* to linkage group II by virtue of its linkage to dilute (*d*)[9]. In a more recent report by these authors, the gene order *Mod-1—d—Thy-1* was established by a three-point cross[10]. The tentative order *Thy-1—d—Mod-1—lu* was suggested on the basis of earlier two-point recombination data for *d—Mod-1*, and *Mod-1—lu*[8], which had indicated that the *Mod-1* locus resided between the other two markers. Shreffler[22], making use of recombination data for *Trf-d* (12.7 percent) and *Trf—lu* (22.2 percent), located *Trf* distal to *d* with respect to the centromere. If *Thy-1* were also distal to *d*, it would necessarily lie close to *Trf*. Such placement of *Thy-1* was also suggested by the data of Lilly who ordered the loci with respect to *Fv-2*[13]. The order *Fv-2—d—Thy-1* with *Fv-2* proximal to *d*, was based on recombination results from two experiments, one using *Fv-2* and *tk*, and the other *Fv-2*, *d*, and *Thy-1*.

Our study demonstrates that *Trf* and *Thy-1* are not closely linked, as would be predicted from the previous studies and the current map. The observed recombination frequency for *Trf* and *Thy-1* is 36–40 percent. Furthermore, our gene order, *Trf—Mod-1—Thy-1*, may be combined with the order *Mod-1—d—Thy-1*, established by Itakura *et al.*, to provide the unambiguous sequence *Trf—Mod-1—d—Thy-1*. The tentative placement of *Trf* and *Thy-1* on the same side of *d* is therefore unacceptable. It remains uncertain, however, what the orientation of this sequence is with respect to the outside markers ducky (*du*) and luxoid (*lu*).

To resolve this issue with a three-point cross, the outside marker must be chosen carefully, because much of the uncertainty regarding chromosome 9 is due to the nature of the markers themselves. For example, *tk*, *du*, *lu*, and *se* have all been reported to depress viability greatly in the homozygous state. Mice homozygous for staggerer (*sg*), *lu*, and *du* are partially or totally sterile, while low penetrance and poor expressivity present additional problems with *lu*[5,7,24]. The orientation of the *Trf—Mod-1—d—Thy-1* sequence might be resolved by a three-point cross with curly whiskers (*cw*) or another outside marker not affected by the problems mentioned above.

The influence of sex on observed recombination frequency in the region between *d* and *Trf*, exemplified in this report as well as in that of Shreffler[22], should be noted. Significantly higher recombination in females has not been reported in the region proximal to *d;* it is likewise not found in our animals between *Mod-1* and *Thy-1*. The distortion of recombination frequencies by sex differences is found often in mice, but the process by which it occurs is not understood[1]. The chromosomal regions where higher female crossing over is observed fit into no obvious pattern, and even two studies of the same region may not agree on the presence of sex distortion of the recombination fraction. The possibility of such recombination differences, especially where loci are being ordered by two-point crosses, may tend to obscure the true sequence of markers in chromosome 9. The linkage map of this chromosome should be used with some caution until the proper sequence of existing markers is elucidated.

Summary

Reciprocal crosses were made between mice of strains CBA/J and RF/J. Segregation of the chromosome 9 markers *Thy-1*, *Mod-1*, and *Trf* was followed by means of backcrosses to the parental strains. These three-point data, combined with two-point data for *Trf-Mod-1* and *Trf-Thy-1*, are consistent with the following map: *Trf*———14———*Mod-1*———23———*Thy-1*. Although the order *Trf—Mod-1—d—Thy-1* is adequately established, the orientation of this sequence with respect to the centromere remains in doubt.

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* Chromosome numbers, rather than linkage groups, are used in the symbols for translocations in accordance with the recommendation of the Committee On Standardized Genetic Nomenclature For Mice [3].

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III. Variation among Sublines of AKR Mice

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Variations among Sublines of Inbred AKR Mice

THE AKR inbred line of mice, developed as a strain with a high incidence of leukaemia, has found wide use in the fields of genetics, immunology, virology, and cancer research. While a certain degree of variability is expected among sublines derived from any inbred strain, rather striking differences have been observed among AKR sublines. Because such differences can be crucial to the usefulness of AKR mice for biomedical research, observations regarding some of these AKR sublines have been collected from several laboratories.

Figure 1 shows a partial history of some of the AKR sublines examined here. The AKR strain was originally developed as "Ak" by Jacob Furth between 1928 and 1936[1]. Eventually, descendants of this stock were sent to Oak Ridge National Laboratories by Dr Lynch, where they were maintained by random breeding and brother × sister matings. Furth, incidentally, may have brought some of his own Ak stock from Dallas to Oak Ridge in 1949. This would place the original separation of the AKR/J stock from the Oak Ridge pool of AKR mice at either 1936 or 1948.

Serological test results for mice of the AKR sublines J and Cum are summarized in Table 1[2-6]. Of particular note is the observation that sublines AKR/J and AKR/FuRdA express the Thy-1.1 thymus antigen (formerly called θ-AKR)[7] while AKR/FuA and AKR/Cum (ref. 8) express Thy-1.2 (θ-C3H). All the AKR sublines bearing the Thy-1.2 antigen, including AKR/Ka, were probably obtained from the Oak Ridge collection of AKR mice.

Data for several biochemical markers are given in Table 2. A critical observation is that the sublines differ at *Mod-1*. This gene, which governs the expression of malic enzyme, is located on chromosome 9 (LG II) approximately twenty map units from *Thy-1*[9,10]. Data on the expression of MuLV-gs antigen by several of the AKR substrains are presented in Table 3. The AKR/FuA subline, which has a relatively low incidence of spontaneous leukaemia, also shows decreased expression of the MuLV-gs antigen. In contrast, mice of the high-leukaemic AKR/FuRdA substrain were invariably MuLV-gs$^+$ as judged by immunofluorescence[11] and *in vivo* absorption tests[12,13], even when foster nursed to MuLV-gs$^-$ mothers of strains C3Hf or GR.

Although the AKR line was originally developed as a high-leukaemic line, the available sublines vary considerably in this respect. For example, the average incidence of leukaemia in mice of subline AKR/FuRdA (generations 5-18) was 92%

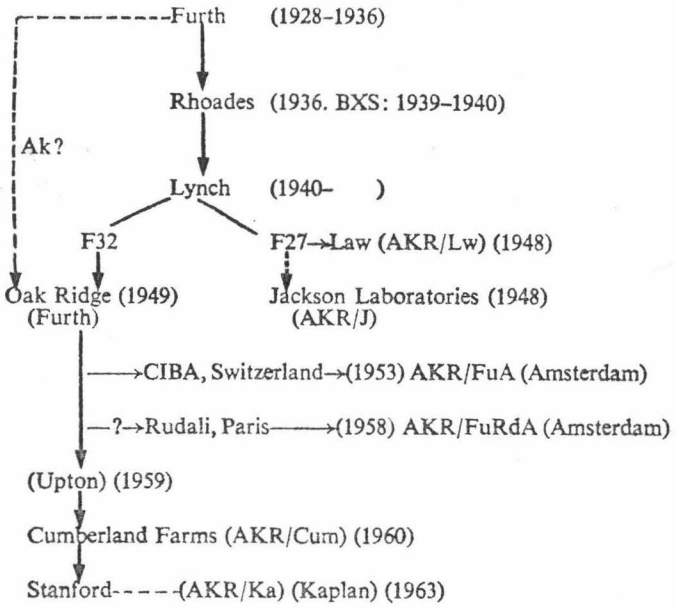


Fig. 1 Lineage of AKR mice.

(273/297) at age 9 months. Since the period 1959-1963, when these data on subline AKR/FuRdA were collected, this subline has continued to show a high incidence of spontaneous leukaemia. On the other hand, mice of subline AKR/FuA, during generations 16-36, showed a leukaemia incidence of only 36% (109/303) at age 11 months. A sister subline, F^m, developed leukaemia at a rate of 20% (45/227) at 12 months for generations 21-35. Preliminary data suggest that the AKR/Cum subline may show a lower spontaneous leukaemia incidence than AKR/J, but larger numbers of mice must be studied over a longer period before this question can be resolved. When combined with the genetic data from Tables 1 and 2, these findings suggest an association between leukaemogenesis and chromosome 9. Such an association has been reported before in the case of Friend virus-induced leukaemias. *Fv*-2, a gene determining susceptibility to this virus, is located on this chromosome[14].

In addition to their variability with respect to spontaneous leukaemias, sublines of AKR also differ in their susceptibility to transplanted leukaemias. Depending on the primary tumour used, resistance to its transplantation may be partial or complete. In an initial experiment, inocula of AKR/J primary leukaemia cells, which were lethal upon transplantation into other AKR/J mice, were successfully rejected by AKR/Cum mice. The only antigen for which the two sublines are known to differ is Thy-1, which may be serving as a histocompatibility antigen. It has been reported that A/Thy-1.1 mice (previously

Table 1 Cell Surface Antigens

Antigen	(LG)*	AKR/J	AKR/Cum
Thy-1.1	(II)	+	—
Thy-1.2		—	+
Ly-1.1	(XII)	—	—
Ly-1.2		+	+
Ly-2.1	(XI)	+	+
Ly-2.2		—	—
Ly-3.1	(XI)	+	+
Ly-3.2		—	—
TL.1,2,3,	(IX)	—	—
H-2^k	(IX)	+	+
$G_{1x}2$		+	+
GCSA		+	+

* LG indicates linkage group.

Table 2 Biochemical Genetic Markers

Marker	(LG)	AKR/J	AKR/FuRdA	AKR/Lw	AKR/Cum	AKR/FuA
Hbb	(I)	d	d	d	d	d
Gpi-1	(I)	a	a	a	a	a
Mod-1	(II)	b	b	b	a	a
Trf	(II)	b	–*	–	b	–
Es-3	(VII)	c	c	c	c	a
Mup-1	(VIII)	a	a	a	a	b
Gpd-1	(VIII)	b	b	b	b	b
Dip-1	(XIII)	b	b	b	b	b
Id-1	(XIII)	b	b	b	b	b
Pgm-1	(XVII)	a	a	a	a	a
Es-1	(XVIII)	b	b	b	b	b

* Strain not tested.

A/θAKR) reject leukaemias from the congenic strain, A/J, which expresses the Thy-1.2 antigen[15].

Preliminary genetic studies using the progeny of (AKR/Cum × AKR/J) F1 mice backcrossed to either AKR parent indicate that a relative resistance to another primary transplanted AKR/J thymoma segregates with the *Thy-1*^{b} genotype. F1 and *Thy-1*^{a} homozygous mice die of the tumour in 17.16 ± 0.52 and 17.42 ± 0.60 d, respectively, whereas Thy-1.1 negative mice (*Thy-1*^{b} homozygotes) die of the leukaemia in 23.75 ± 0.97 d (mean ± s.e.). No association was found between resistance and the *Mod-1* locus product in the several animals also tested for malic enzyme type. In a control experiment, small numbers of AKR/Cum mice were preimmunized with either four injections (intraperitoneal) of 10^7 AKR/J liver cells or four injections of 10^7 AKR/J normal thymus cells. Both groups were

Table 3 Quantitative Immunofluorescence Tests for MuLV-gs Antigen in Spleens of Adult Mice of Various AKR Sublines

Subline	Laboratory	Age in months	No. positive/No. tested IFA*	DR†	Titre IFA	ABU d^{-1}† DR
AKR/J	Sloan-Kettering Institute	1–8	22/22	3/3	1/4–1/16	11–>15
AKR/J	Department of Radiobiology, Stanford	6	2/2		1/8	
AKR/Ka	Department of Radiobiology, Stanford	1–6	12/12		1/4–1/16	
AKR/FuRdA	Netherlands Cancer Institute	1–9	32/32	9/9	1/4–1/16	10–>15
AKR/FuRdAfC3Hf‡	Netherlands Cancer Institute	1		1/1		15
AKR/FuRdAfGR‡	Netherlands Cancer Institute	2	3/3		1/4–1/8	
AKR/FuA	Netherlands Cancer Institute	2–5	0/8	3/10		10, 13, 15
AKR/FuA	Netherlands Cancer Institute	11–18	2/6	3/4	1/1–1/4	8, 10, >15

* IFA, Immunofluorescence absorption. The antigen titre refers to one volume of a 17% wet weight extract in 0.85% saline, which absorbs out the immunofluorescence of an equal volume of antiserum, diluted to three double dilutions below the endpoint of the fluorescence (for example, 1/64 if the endpoint of fluorescence is 1/512).

† DR, Disappearance rate. ABU d^{-1} refers to the disappearance rate per day of intraperitoneally injected rat anti-MuLV-gs antibodies from the serum of adult mice as measured by immunofluorescence. A disappearance rate of less that 2 ABU d^{-1} is considered negative.

‡ Foster nursed to MuLV-gs mothers of strain C3Hf or GR, as indicated.

able to reject completely a challenge inoculation of 5×10^6 Thy-1.1 positive AKR/J spontaneous thymoma cells, whereas the unimmunized AKR/Cum mice died of leukaemia within 23 d. Because liver preparations are generally considered Thy-1 negative[2], and because no cytotoxic anti-Thy-1.1 antibodies were found in the sera of animals preimmunized only with liver cells, further work is in progress to determine the effect(s) of factors other than Thy-1 antigenicity in this immune rejection phenomenon.

In summary, sublines of the inbred AKR mouse strain have been found to differ with respect to several traits, including the Thy-1 antigen, malic enzyme, esterase-3, principal urinary protein, expression of MuLV-gs antigen, and incidence of leukaemias. Within the context of these AKR sublines, the data suggest an association between chromosome 9 and leukaemogenesis. Further research in several fields will be required to test this hypothesis. For the present, researchers using AKR mice should be alerted to the subline variations reported.

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IV. Immunogenetics of a Thymus Antigen in Lymphoma-Prone and Lymphoma-Resistant Colonies of Wild Mice

The publication contained in this chapter originally appeared in the Journal of the National Cancer Institute

Immunogenetics of a Thymus Antigen in Lymphoma-Prone and Lymphoma-Resistant Colonies of Wild Mice [1,2]

Elizabeth P. Blankenhorn,[3] Murray B. Gardner,[4] *and* John D. Estes [5,6]

SUMMARY—The Thy-1 (theta) antigen was identified in wild mice (*Mus musculus*), the frequency of its alleles was determined in two natural populations of wild mice, and the possible T-cell origin of spontaneous lymphomas was investigated in one of these populations. Reaction patterns for Thy-1 antigen with the use of direct cytoxicity and indirect absorption assays were identical in wild mice and inbred strains. Between 15 and 55% of viable spleen cells from healthy young or old wild mice were Thy-1 positive. Spleen, but not brain, cells from older wild mice were less strongly positive. Wild mice from the lymphoma-prone population were polymorphic for *Thy-1*^{a} and *Thy-1*^{b} alleles, whereas wild mice from the lymphoma-resistant populations were homozygous for the *Thy-1*^{b} allele. Contrary to expectation, a higher frequency of the *Thy-1*^{b} allele was noted in the mice with lymphomas. Alleles at two other loci on chromosome #9 (*Mod-1*^{a} and *Trf*^{b}) were fixed in both populations. The absence of detectable Thy-1 antigen on spleen cells (despite its detectability in undiminished titer in brain tissue) in 80% of mice with lymphomas, along with the absence of thymus involvement in the lymphomatous proliferations, suggested that these tumors are derived from an expansion of non-thymus-derived cells.—J Natl Cancer Inst 54: 665–672, 1975.

THE THY-1 (formerly theta) antigen is a useful marker in laboratory mice for distinguishing lymphoid cells of thymic origin (T cells) (*1*). Two codominant alleles (*a* and *b*) determine the phenotypic expression of *Thy-1* in laboratory mice (*2*). The *Thy-1*^{a} allele controls an antigen designated Thy-1.1; the *Thy-1*^{b} allele controls the alternate antigen, Thy-1.2 (*3*). The detection and distribution of these alleles have not been reported in wild mice. This paper shows that Thy-1 can be detected in normal wild mice (*Mus musculus*) and compares the frequencies of alleles at *Thy-1* and two other loci (*Mod-1* and *Trf*) on chromosome #9 in two natural populations of wild mice differing markedly in their incidence of spontaneous lymphoma. It also investigates the possibility that the lymphomas observed were of T-cell origin.

MATERIALS AND METHODS

Wild mouse colonies.—The trapping areas and methods were described in (*4*). Wild mice from trapping area #1 (colony #1), upon aging in captivity, were generally resistant to spontaneous cancer and latent type-C virus activation, despite a high prevalence of indigenous polyoma virus infection (*5*). Wild mice from area #3 (colony #3), upon aging in captivity, were unusually prone to spontaneous lymphoma and had frequent leukemia and a motor neuron type of lower limb paralysis, both attributable to a high level of indigenous type-C virus activity (*6*, *7*). During the first year of observation, the rate of lymphoma and paralysis in this colony was fairly constant at ≈1% per month among the surviving mice.

Tissues were obtained from freshly trapped normal young adult mice from colony #3, from young first-generation laboratory-bred mice from colony #3, from aging normal mice from colony #1 held in captivity, and from aging mice from colony #3 having or not having developed spontaneous lymphoma while under laboratory observation. A complete necropsy and a histopathologic study by routine procedures (*4*) were done on each mouse, and the diagnosis of lymphoma was confirmed microscopically. The age of the mouse was estimated from the weight of the animal when captured and the period of observation (*4*). Mice less than 18 months of age were considered "young" and those over 18 months of age were "old."

Selection and preparation of tissues.—Brains and lymphoid organs were removed from each mouse immediately after sacrifice. The brains were frozen quickly on dry ice and thawed immediately before use. Spleens were taken from every mouse, but lymph nodes were taken mainly from the mice with lymphomas because the nodes were often too tiny for study in the normal mice. Thymuses were taken only from recently trapped young mice; in older mice, including those with lymphoma (*6*), the thymus was markedly atrophic. After a small piece of spleen or lymph node was removed for light microscopy, the remaining tissue was placed in individual sample bottles containing Eagle's minimum essential medium with 2% fetal calf serum (FCS) and kept on ice. We prepared single cell suspensions by teasing the tissue gently with scalpels in a small quantity of medium, aspirating the suspension up and down in a Pasteur pipette, and taking the supernatant after allowing the clumps to settle out. The cell suspensions were then centrifuged at 500×*g* for 5 minutes, and the medium was replaced with Hanks' balanced salt solution (HBSS) supplemented with 2% FCS and 1% antibiotic-antimycotic solution concentrated 100 times (Gibco, Santa Clara, Calif.). Nucleated cells were counted in a hemacytometer.

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This counting was facilitated in later experiments by the dilution of an aliquot of the cell suspension in 0.01% crystal violet in 0.1 M citric acid. Plasma was obtained from some animals by centrifugation of heparinized blood samples.

Thy-1 typing.—AKR/J anti-C3H/HeJ (anti-Thy-1.2) and C3H/HeJ anti-AKR/J (anti-Thy-1.1) antisera were prepared by injection of thymocytes into the reciprocal strain according to the schedule reported by Reif and Allen (*1*). Unless otherwise noted, all typing was performed using the serum obtained from one bleeding. Two methods were employed to type each animal for Thy-1 antigen.

Direct cytotoxicity.—Lymphoid cell suspensions were adjusted to 2×10^6 cells/ml of supplemented HBSS. A modification of the two-step method of Amos et al. (*8*) was used, in which 1 μl of serially diluted antiserum was dispensed into each well of a #3034 microtest plate (Falcon Plastics, Division B-D Laboratories, Inc., Los Angeles, Calif.) and 1 μl of the cell suspension to be tested was then added. After incubation for 15 minutes at room temperature, $\approx$10 μl of HBSS was added to wash out the excess antibody. After this wash was removed, 1 μl of rabbit complement [diluted 1/9 in HBSS following agarose-EDTA absorption (*9*, *10*)] was added to the sensitized cells; the plate was further incubated for 20 minutes at 37° C in a 6% CO_2 atmosphere. Into each well was dropped 10 μl of 0.3% trypan blue in HBSS, and dye exclusion was used to assess the percentage of cells killed by antiserum and complement. Controls for complement toxicity (in which medium was substituted for antiserum) were included for every sample tested, and all tissue samples were tested in duplicate.

Absorption method.—Each brain was weighed upon thawing and homogenized in HBSS with the use of tissue grinders of the Potter-Elvehjem type to make a 100 mg/ml (wet wt) suspension. After centrifugation at $700\times g$ for 10 minutes at room temperature, the supernatant was removed for *Mod-1* classification and replaced with fresh HBSS. Aliquots containing 0.5, 1.0, and 5.0 mg were made for Thy-1.2 typing; those containing 0.05, 0.1, and 0.5 mg were prepared for Thy-1.1 typing. They were centrifuged in small plastic tubes at $\approx1{,}000\times g$ for 5 minutes, the supernatants were discarded, and the pellets were dispersed vigorously in 50 μl of diluted antiserum added to each tube. Dilutions of antiserum were chosen that were twice the concentration giving 50% cytolysis with the the use of inbred mouse thymocytes in the direct cytotoxicity test. After 30 minutes' incubation at room temperature, the absorbed antiserum was removed by centrifugation and stored individually at −20° C for subsequent typing by the direct cytotoxicity method.

Absorptions were performed with graded numbers of washed spleen and lymph node cells in the same manner as described for brain tissue. Often the number of lymph node cells from normal wild mouse donors was limiting because of the small nonlymphomatous lymph nodes.

Preliminary studies on 8 normal mice from colony #3 proved that similar results could be obtained with the absorbed antisera and either self-targets or inbred mouse thymocytes, which demonstrates that inbred mouse thymocytes may be used as target cells to assay for the presence of Thy-1 on wild mouse tissues. This is important because suitable wild mouse thymocytes (self-targets) are difficult to obtain from the atrophic thymuses of aged or ill mice, which constitute the majority of subjects in this work.

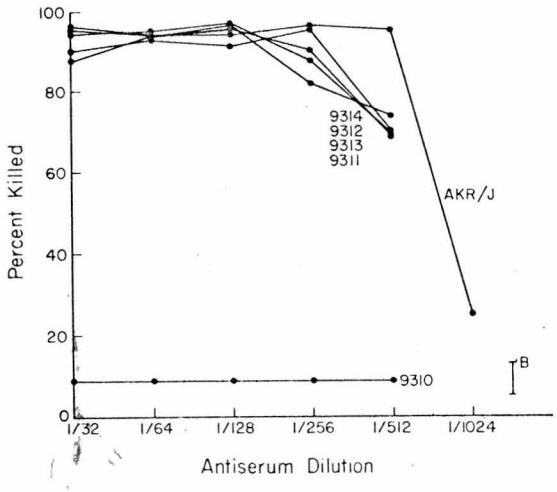
Evaluation of Thy-1-typing results.—Results from direct typing were expressed graphically as percent killed at each of five serial dilutions of anti-Thy-1 antiserum. Results from the direct cytotoxic assessment of absorbed antiserum were expressed in terms of the amount of tissue required for absorption of 50% of the cytotoxic activity of the antiserum employed (*9*, *11*).

Mod-1 and *Trf.*—Procedures for malic enzyme and transferrin typing by starch gel electrophoresis are in (*12*, *13*).

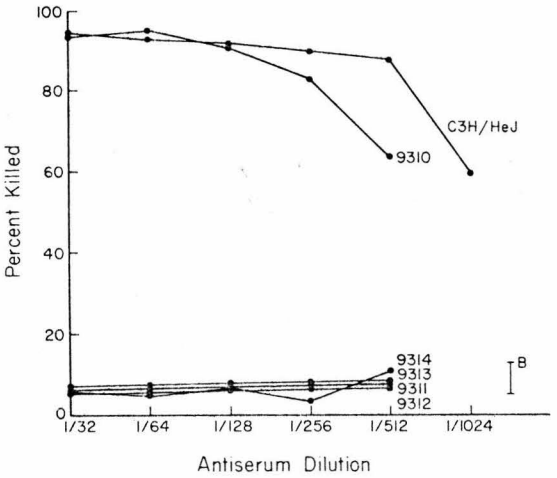
RESULTS

Young healthy wild mice may be easily typed for Thy-1 by assay of the direct cytotoxic activity of anti-Thy-1 antiserum on fresh thymus cells in the presence of complement. Fifteen normal young mice from laboratory-bred colony #3 were tested in this manner, and reaction patterns to dilutions of anti-Thy-1 antiserum were identical to those found for inbred strains (text-figs. 1, 2). Lymphoid cells from the spleens and lymph nodes of healthy mice could also be typed reliably by this method.

Absorptions of anti-Thy-1 antisera by brain tissues from mice in colonies #1 and #3 also gave results similar to those with inbred mouse strains. All brain samples were positive for either Thy-1.1 or Thy-1.2, or both. Brain-tissue typing may be considered definitive for demonstration of the presence of Thy-1 antigens (*14*), so the genotypes for this codominantly



TEXT-FIGURE 1.—Killing of wild mouse and AKR/J thymocytes by C3H/HeJ anti-AKR/J (anti-Thy-1.1) serum. Individual wild mouse samples are indicated by mouse number. B=range of background cell death without antiserum.



TEXT-FIGURE 2.—Killing of wild mouse and C3H/HeJ thymocytes by AKR/J anti-C3H/HeJ (anti-Thy-1.2) serum. Individual wild mouse samples are indicated by mouse number. B=range of background cell death without antiserum.

expressed pair of alleles could be inferred from the results of data on brain absorption.

Genotypes for 57 mice from colony #3 were determined by both direct cytotoxicity and brain absorption methods. Fifteen of these mice (obtained from 4 unrelated litters in the laboratory breeding colonies) were normal siblings, 17 were normal mice trapped in area #3, and 25 were mice with lymphomas and also trapped in the wild. The observed frequency of the *Thy-1*^{a} and *Thy-1*^{b} alleles was 0.43 and 0.57, respectively. The calculated frequency of heterozygotes in Hardy-Weinberg equilibrium would be 0.49, and the observed frequency in the colony #3 population was 0.40 (chi-square = 1.8; $P > 0.10$). In colony #3 mice bearing spontaneous lymphomas, however, the frequency of the *Thy-1*^{b} allele was significantly higher (0.70 in the mice with lymphomas vs. 0.47 in the normal colony #3 mice; chi-square = 6.98; $P < 0.05$).

Twenty healthy older mice were examined from colony #1; all were homozygous for the *Thy-1*^{b} allele. Thirteen of these mice were taken from 3 unrelated litters; the remainder were not known to be related.

No polymorphism for the other chromosome #9 markers was found in either colony. Twenty-four colony #3 mice, of which 8 were known Thy-1 heterozygotes, and 8 colony #1 mice tested had the *Mod-1*^{a} allele. The transferrins of all of the 15 colony #3 mice, including 4 Thy-1 heterozygotes, and 8 colony #1 mice were of the type controlled by the *Trf*^{b} allele.

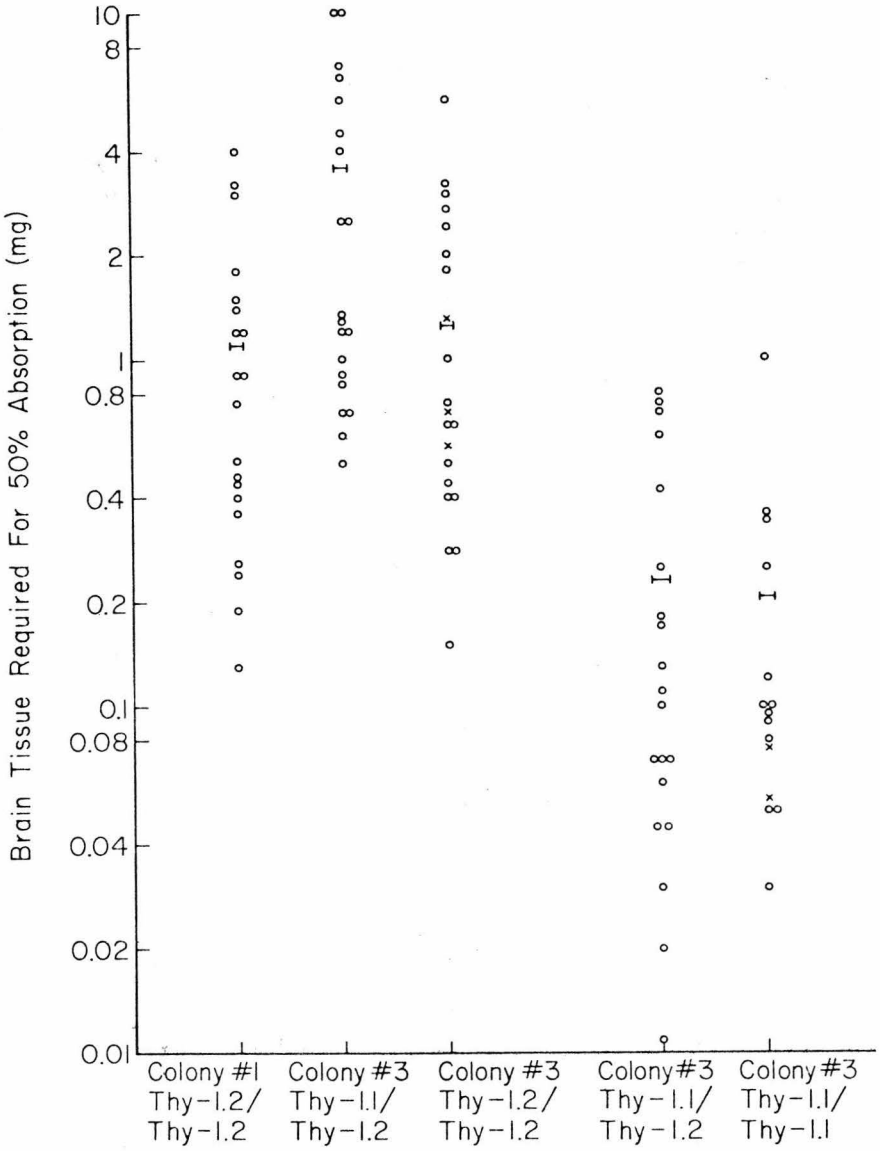
Absorption patterns from wild mouse brain tissue were generally similar to those from inbred mouse brain tissue (text-fig. 3). Some variation was detected, however, between experiments; therefore, both brain- and lymphoid-tissue-absorbed antisera were tested simultaneously and never allowed to thaw twice. In deciding the genotype of an animal, we considered concordance between normal lymphocyte and brain tissue absorption results as being important. Brain tissue absorptions were considered negative if the values were discontinuously in excess of those shown (for Thy-1-positive results) in text-figure 3, i.e., greater than 10 mg for the anti-Thy-1.2 reagent and greater than 1 mg for the anti-Thy-1.1 serum. The anti-Thy-1.2 reagent (at 1/400) distinguished between homozygotes and heterozygotes for the corresponding *Thy-1*^{b} allele; anti-Thy-1.1 (at 1/256) did not detect the difference between homozygotes and heterozygotes for the corresponding *Thy-1*^{a} allele. Thy-1 antigen representation on brain tissue did not vary with mouse age or sex (table 1) or trapping area (text-fig. 3). Furthermore, brain absorption values were statistically the same for healthy mice and those bearing lymphomas (table 2), which demonstrates that brain absorptive capacities had not deteriorated in mice with lymphomas.

Studies with normal wild mice from both colonies and inbred mice demonstrated that Thy-1 antigen could be detected on spleen cells by complement-dependent, antibody-mediated cytotoxicity. Between 15 and 55% of the viable spleen cells from most healthy old (>18 mo) or young wild mice were killed by a 1/16 dilution of either appropriate antiserum. Spleen cells from older mice were less susceptible to killing by anti-Thy-1 serum plus complement; however, 12 of 19 of these did show killing greater than 15% above background. In only 2 of the 52 assays performed on 26 normal spleen specimens was there significant killing of cells by the reagent that would not have been expected, from the brain absorptions, to have been active. Failure to detect significant killing by the appropriate antiserum was found in only 4 of the 52 assays. These 4 assays were performed on 2 mice with reactive hyperplasia of the spleen, characterized by plasma cell (Thy-1 negative) proliferation, and on 2 rather old mice (29 and 30 mo old). All 4 "negative" normal spleens, however, could absorb Thy-1 antibodies (*see* below) in subsequent testing.

TABLE 1.—*Absorption of anti-Thy-1 serum by brain tissues of male and female wild mice* [a]

Mice	Absorption of anti-Thy-1.1		Absorption of anti-Thy-1.2	
	Homozygotes	Heterozygotes	Homozygotes	Heterozygotes
Males (17)	0.23±0.09(2)	0.11±0.03(5)	1.64±0.54(10)	1.97±1.12(5)
Females (36)	0.21±0.09(10)	0.24±0.10(8)	1.22±0.26(18)	1.92±0.55(8)

[a] Values are the means and SE of milligrams of brain required to give 50% absorption of the reference reagent; numbers of mice studied are *in parentheses*. Mice from both colonies are included.



TEXT-FIGURE 3.—Brain tissue absorptive capacities for homozygous and heterozygous mice from colony #3 and from homozygous mice from colony #1. *First three columns* contain values of Thy-1.2 in brains of individual mice; *last two columns* contain values of Thy-1.1. *Bars* represent mean absorptive capacities of wild mouse brains in each group; x=inbred mouse (C3H/HeJ and C57BL/6J for Thy-1.2, AKR/J for Thy-1.1) brain absorptive capacities.

TABLE 2.—*Absorption of anti-Thy-1 serum by brain tissues of healthy wild mice and wild mice with lymphomas* [a]

Mice	Absorption of anti-Thy-1.1		Absorption of anti-Thy-1.2	
	Homozygotes	Heterozygotes	Homozygotes	Heterozygotes
Total (71)	0. 21±0. 07(13)	0. 23±0. 06(20)	1. 29±0. 20(38)	3. 55±0. 93(20)
Lymphoma-bearing (30)	0. 29±0. 14(6)	0. 18±0. 08(9)	1. 66±0. 38(15)	2. 88±1. 18(9)

[a] Values are expressed as in table 1 and include data from both colonies.

TABLE 3.—*Absorption results from spleens of wild mice* [a]

Mouse No.	Diagnosis	Antigen studied	Number of spleen cells $\times 10^6$ required for 50% absorption	Results of direct test [b]	Brain absorption [c]
11173	Normal [d]	Thy-1.1	8.0	−	0.70
11205	Normal	Thy-1.1	1.5	−	.20
6617	Normal	Thy-1.1	7.0	±	.07
6187	Pulmonary adenoma	Thy-1.1	3.0	±	.10
4377	Normal	Thy-1.2	5.0	nt	.28
11262	Normal	Thy-1.1	15.0	nt	.16
4606	Normal	Thy-1.2	5.0	+	.24
3512	Normal	Thy-1.2	5.0	+	.36
3879	Normal	Thy-1.2	3.6	−	.75
4515	Normal	Thy-1.2	3.4	+	1.20
5804	Normal	Thy-1.2	2.5	−	0.51
3701	Normal	Thy-1.2	3.2	+	.13
9574	Normal	Thy-1.2	7.0	+	1.80
9575	Normal	Thy-1.2	21.0	nt	1.20
9576	Normal	Thy-1.2	11.0	+	0.86
9577	Normal	Thy-1.2	21.0	+	1.35
9578	Normal	Thy-1.2	16.0	nt	0.90
9615	Normal	Thy-1.2	11.5	+	.46
9616	Normal	Thy-1.2	11.5	+	3.00
9617	Normal	Thy-1.2	6.5	+	0.19
12837	Normal	Thy-1.2	6.5	+	3.20
6311	Hepatoma	Thy-1.1	6.2	nt	0.05
		Thy-1.2	2.2	nt	1.20
6543	Pulmonary adenoma [e]	Thy-1.1	>60	±	0.10
		Thy-1.2	>40	±	4.00
7307	Pulmonary granuloma [e]	Thy-1.1	>20	+	0.17
		Thy-1.2	>20	+	4.50
6279	Lymphoma [f]	Thy-1.1	5.0	nt	0.05
		Thy-1.2	16.0	nt	.71
6799	Lymphoma [f]	Thy-1.2	5.0	−	2.00
6365	Lymphoma [f]	Thy-1.2	8.5	−	0.15
7310	Lymphoma [f]	Thy-1.1	7.0	−	.05
10493	Lymphoma [g]	Thy-1.2	11.0	−	.44
10162	Lymphoma [f]	Thy-1.2	3.2	+	.65
11125	Lymphoma [f]	Thy-1.1	3.0	+	.05
10806	Lymphoma [f]	Thy-1.1	> 7	−	.07
7179	Lymphoma [h]	Thy-1.1	>40	−	.07
7497	Lymphoma [f]	Thy-1.2	>10	−	.50
10636	Lymphoma [f]	Thy-1.2	>10	±	1.00
7400	Lymphoma [f]	Thy-1.1	>20	−	0.13
		Thy-1.2	>20	−	1.35
11072	Lymphoma [g]	Thy-1.1	>20	nt	0.80
		Thy-1.2	>20	nt	1.30
7437	Lymphoma [f]	Thy-1.2	>10	+	3.20
7247	Lymphoma [f]	Thy-1.2	>35	−	2.70
10715	Lymphoma [f]	Thy-1.1	>25	−	0.10
10587	Lymphoma [f]	Thy-1.2	>20	−	.28
11103	Lymphoma [h]	Thy-1.1	>25	−	.01
11401	Lymphoma [f]	Thy-1.1	>20	nt	.03
		Thy-1.2	>20	nt	.90
10266	Lymphoma [f]	Thy-1.1	>20	nt	.06
		Thy-1.2	>22	nt	2.50

[a] The number of spleen cells required to absorb 50% of the activity of the appropriate anti-Thy-1 serum was calculated (*see* text) for 22 normal mice from colonies #1 and 3, 20 lymphoma-bearing mice from colony #3, and 2 mice from colony #3 which may have had early lymphomas. Negative results are expressed as "greater than" the maximum number of cells used for absorption study. Results from the cytotoxic test on each spleen and from the absorption tests of the brain of each mouse are included for reference.

[b] −, 10% killed over background; ±, 10–15% killed; +, 15–55% killed; nt, not tested.

[c] Results are expressed as mg of brain required for 50% absorption of the appropriate antiserum.

[d] All animals without malignancy are referred to as "normal."

[e] Possible early lymphoma.

[f] Common malignant lymphoblastic lymphoma found in colony #3 mice.

[g] Reticulum B cell lymphoma.

[h] Probable histiocytic lymphoma.

In contrast to normal spleens, cells from 80% (16/20) of the lymphomatous spleens tested were not killed by either anti-Thy-1 serum and complement. This is unlike the pattern seen in AKR/J mouse thymic leukemias. Most AKR/J spleens contain a high percentage of Thy-1-positive cells resulting from the malignant expansion of thymus-derived clones and their peripheralization (*15*).

The absence of positive cytotoxicity in the wild mice with lymphomas as seen in the direct cyto-

TABLE 4.—*Summary of data on spleens of wild mice*

Measurement	Nonlymphomatous spleens		Lymphomatous spleens: Colony #3
	Colony #1	Colony #3	
Number positive/No. tested for Thy-1 antigen:			
By absorption	15/15	7/9	7/20
By cytotoxicity	11/13	11/13	4/20
Number negative/No. tested for Thy-1 antigen:			
By absorption	0/15	2/9	13/20
By cytotoxicity	2/13	2/13	16/20
Spleen weight:			
Average	0. 07 g	0. 13 g	0. 67 g
Range	0. 03–0. 21 g	0. 02–0. 97 g	0. 21–1. 50 g
Average period of observation (range)	9 mo (0–24 mo)	18 mo (8–26 mo)	17 mo (9–25 mo)

toxicity assay was also reflected in the absorption tests. With two exceptions, between 2×10^6 and 20×10^6 spleen cells from normal wild mice could specifically absorb 50% of the activity of the appropriate anti-Thy-1 antiserum (table 3). Based on histopathology, the two exceptions ("normal" animals from colony #3 providing spleen cells with no detectable Thy-1 antigen) could have had early malignant lymphomatous involvement of the spleen. In contrast to the normal wild mice, 13 of the 20 tested mice with lymphomas failed to show significant absorption of the appropriate antiserum by spleen cells (table 3). Seven leukemic spleens exhibited some Thy-1 antigen, and 4 of these appeared to be strongly Thy-1 positive. Three of these 4 samples were also positive (18% killing over background) when tested by the direct cytotoxicity assay. Of the remaining 4 absorption-positive leukemic mice, 2 did not show positive direct typing results, and 2 were not tested by this method. The results from experiments with wild mouse spleen cells are summarized in table 4.

There were no gross or microscopic differences in the lymphomatous pattern, distribution, or histopathology between Thy-1 positive and Thy-1-negative lymphomas. None of the lymphomas appeared to arise in or invade the thymus.

Lymph node cells from a few wild mice were tested for Thy-1 antigen. Eleven lymphoma-bearing mice from colony #3 provided lymph node cell samples suitable for direct testing, and seven (63%) of these samples were killed by the appropriate Thy-1 reagent. The percentage of lymphomatous lymph node cells killed, however, never exceeded 25% above background, and four samples showed no clear cytotoxicity. In contrast, all of 12 normal mice from both colonies exhibited significant lymph node cell death in the presence of the appropriate reagent, and the average specific cytotoxicity was 33% (range 15–65%).

Despite our inability to demonstrate that lymphomatous lymph nodes contained strongly Thy-1-positive cells by direct cytotoxic testing, all lymph nodes tested by absorption (from 3 normal mice from colony #1 and 12 mice with lymphomas from colony #3) were shown to have equivalent amounts of Thy-1 antigen. However, only three of the lymph node cell samples shown weakly reactive to anti-Thy-1 serum and complement could provide enough cells to be further tested by the absorption procedure, so the relationship of positive reactions found in the direct test to values obtained by the absorption method cannot be evaluated fairly from these data.

DISCUSSION

These results show that the Thy-1 antigens were demonstrable on appropriate wild mouse tissues by two methods: direct cytotoxicity and absorption. The amount of antigen (Thy-1.1 or Thy-1.2) found in brain tissue was generally comparable to that found in inbred mice. The existence of additional possibly related Thy-1 antigens in wild mice is currently being explored with the use of an alloantiserum (kindly provided by Dr. Ian McKenzie, Massachusetts General Hospital) which is thought to detect other Thy-1 allele products (MacKenzie I: Personal communication). As in inbred mice (*16*), the amount of Thy-1 antigen detected by the cytotoxicity assay on wild mouse spleen cells declines with age, but in most of our experiments on aged mice, Thy-1 expression was not below the limits of detection. The presence of Thy-1 antigens on normal lymphoid cells could be confirmed by absorption experiments which, as with brain tissue, demonstrated the antigen in amounts equivalent to those in inbred mice.

Allele frequencies for the three markers on chromosome #9 were calculated for both colonies. The Trf^b, $Mod\text{-}1^a$, and $Thy\text{-}1^b$ alleles were fixed in colony #1 mice. Colony #3 mice also demonstrated fixation of the $Mod\text{-}1^a$ and Trf^b alleles, but were polymorphic for the *Thy-1* locus. Genotypes of this locus were in Hardy-Weinberg equilibrium. It is interesting that the $Thy\text{-}1^a$ allele was segregating in this colony #3 population, because this allele has been previously noted to show some coincidence with the occurrence of leukemias in different sublines of AKR mice (*17*). However, the $Thy\text{-}1^a$ allele was *not* found to have a higher frequency in the group of colony #3 wild mice with proved lymphomas, as compared with age-matched mice from the same colony without lymphomas. Therefore, the presence of this allele cannot yet be associated with the predisposition to lymphoma in these wild mice. There is no obvious explanation for the polymorphism of *Thy-1* in colony #3.

The absence of Thy-1 antigen detectable within the limits of the absorption assay in most (65%) of

the lymphomatous spleens indicates that the majority of spontaneous lymphomas arising in wild mice from colony #3 were probably not of T-cell origin. The possibility that wild mouse lymphoma cells bear Thy-1 antigens undetectable by absorption, because of poor cross-reactivity with antisera prepared to the Thy-1 antigens of inbred mice, is made unlikely by comparison with the normal values observed for brain Thy-1 absorption by the same lymphomatous donors. This absence of Thy-1 antigen on the lymphoma cells may represent a loss of antigenic expression after transformation of thymus-derived clones, depletion of the recirculating T-cell pool, or the malignant expansion of non-thymus-derived cells, a hypothesis we favor. This hypothesis is supported by the universal absence of thymic involvement in the lymphomatous disease of these mice (*6*).

The lymphomas in these mice did not seem to be of B-cell origin. The lymphomatous proliferation did not arise by expansion of B-cell-dependent lymphoid follicular centers, nor was there histologic evidence of neoplastic plasma cell differentiation. Preliminary results from an immunofluorescent study failed to reveal detectable mouse Ig on the surface of spleen lymphoma cells or on cultured lymphoid cells derived from several colony #3 mice with lymphomas (Nies K: Unpublished observations).

The seven Thy-1 antigen-positive lymphomatous spleen cell populations cannot be conclusively categorized as T-cell lymphomas, because the assay methods do not distinguish between large amounts of antigen on a few cells and small amounts of antigen on many cells. A population of normal T cells, intermingled with Thy-1-negative lymphomatous cells in the spleen, could therefore give the results observed in these seven cases. Consistent with this possibility is the fact that these wild mouse lymphomatous spleen cell populations gave weaker tests for Thy-1 antigen than do spleen cell populations in AKR/J mice with thymic lymphomas (Blankenhorn EP: Unpublished observations).

There are a number of difficulties in using the direct cytotoxicity and absorption methods to prove the presence or absence of Thy-1 antigens on lymphoid cells in noninbred wild mice. Neither method can specifically detect the number of antigenic sites on a single cell or on each cell in a given population. Furthermore, the possibility of cross-reactivity (rather than antigenic identity), which may complicate the interpretation of absorption results, can decrease the value of this method. Confidence in an assessment of Thy-1 antigens on lymphocytes of an individual wild mouse requires the concurrent testing of the brain tissue of the mouse. In our studies, use of this technique eliminated questions of significantly weak reactivity. It also reduced the likelihood of being misled by the detection of other antigens such as Ig (*18*) or Ly-1 (*19*), since these antigens are not found on washed brain tissue. There is some day-to-day variation in the results obtained from the absorption assays, and the inaccuracies that can result from refreezing and rethawing absorbed, diluted antiserum prohibit reevaluation of any equivocal data. The absorption procedure for lymphocytes must be done with at least three graded numbers of cells (e.g., 1×10^6, 5×10^6, and 10×10^6), and the total quantity necessary is often difficult to obtain from the lymphoid organs of individual old wild mice. In addition, because of the cytotoxicity-negative, absorption-positive phenomena familiar in this type of experiment (*20*), contradictory results may be found in the two different assays of the same cell population. Fortunately, this condition was rare in the present study.

The absence of T- and B-cell surface markers and of pathologic features clearly suggesting T- or B-cell dependence is similar to the findings reported in 50% of lymphomas examined from inbred mice (*21*) and in many human acute lymphoblastic leukemias (*22, 23*). Further study of this natural wild mouse model, with the use of additional assay techniques for determining T- and B-cell function, may help clarify the nature of this apparent "null" population of neoplastic lymphoid cells.

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V. Partial N-terminal Amino Acid Sequence of Rat Transplantation Antigens

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Partial N-terminal amino acid sequence of rat transplantation antigens

THE major histocompatibility complex (MHC) is a genetic region in mammals that controls various immunologically related phenomena. For example, the major histocompatibility complex of the mouse, the *H-2* complex, can be divided into four major regions—*K*, *I*, *S* and *D*—by recombinational analysis[1,2]. The *K* and *D* regions code for transplantation antigens, cell-surface glycoproteins of 45,000 molecular weight which elicit skin, tissue and tumour graft rejection. The *I* region codes for immune response genes and for the Ia polypeptides, cell-surface glycoproteins composed of heterodimers of approximately 35,000 and 28,000 MW, respectively[3,4]. The *S* region contains structural or regulatory genes that control the levels of certain complement components[5,6]. Functional and genetic analyses of these immunological traits suggest that these apparently disparate functions may be closely linked in the major histocompatibility complex of other mammals as well as the mouse[7]. The major histocompatibility complexes of humans, guinea pigs, rats and mice have been shown to have certain significant structural homologies to one another. In rats, the MHC is designated AgB or H-1 (refs 8 and 9, respectively). To date, at least 13 haplotypes, or combinations of allelic, linked genes within AgB, have been identified, based on data from skin grafting and serological testing (ref. 10 and O. Stark, personal communication). Here we present the partial N-terminal sequences of the transplantation antigens isolated from rats of the AgB4 haplotype. Our results demonstrate that the rat AgB4 transplantation antigen is homologous to those of the mouse, human and guinea pig. The sequence data also suggest that at least two major components are present. These sequences may represent the rat homologues of the mouse *K* and *D* transplantation antigens.

The transplantation antigens coded by the AgB complex are of particular interest because they seem to differ from their mouse counterparts in one fundamental property, namely, the extent of genetic polymorphism which they exhibit. In inbred mice there are at least 11 *K* alleles and 10 *D* alleles which form 25 or more haplotypes. Immunogenetic and biochemical analyses of the mouse transplantation antigens show that there

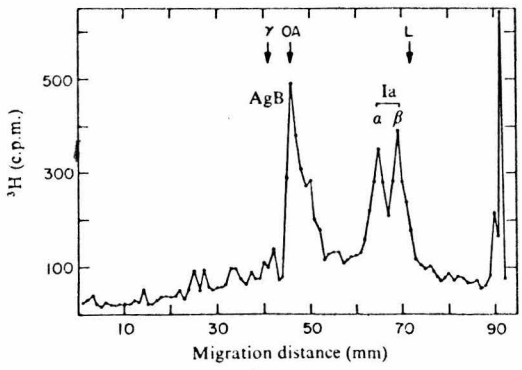


Fig. 1 SDS-PAGE profile of BN.B4 molecules precipitated by (Lewis × BN.B2) F1 anti-DA antiserum. Triton X-100-solubilised antigens from 7.5×10^5 ^{3}H-tyrosine labelled BN.B4 spleen cells were incubated with 20 μl antiserum overnight at 4°C. Antigen–antibody complexes were collected by adsorption to *S. aureus* Cowan 1^{18}, and separated on a 10% gel in the presence of 2-mercaptoethanol. The AgB4 transplantation antigen and Ia-like antigens are shown. Immunoglobulin heavy chain, light chain, and ovalbumin markers are shown by arrows.

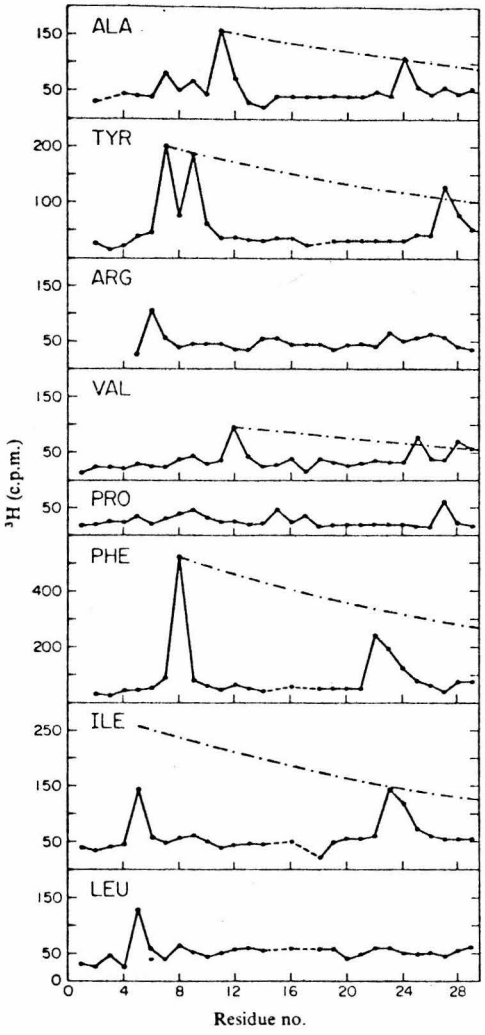


Fig. 2 Microsequence analysis of AgB4. The amount of radioactivity associated with each of the seven incorporated amino acids is plotted against residue number. Repetitive yields (-·-·-·-) are calculated where two or more residues are identified. The isoleucine profile shows residues of roughly equal yields at positions 5 and 23. The simplest explanation for this anomaly is that there are two major sequences being analysed and both sequences have isoleucine at position 23, whereas only a single sequence has isoleucine at position 5. Hence, the repetitive yield curve is drawn from the residue at 23, presumably shared by both major sequences. The chromatography gradient failed to separate adjacent residues at several positions (alanine at position 2, tyrosine at 18, and phenylalanine, isoleucine and leucine at positions 15 and 17). These uncertainties are indicated by broken lines and no residue assignments are made at these positions. The minor peaks for alanine at positions 9 and 11 arise as spillover from the adjacent tyrosine peaks in the chromatography separation. Arginine co-chromatographed with phenylalanine in positions 1–4, and subsequently it was resolved from other labelled amino acids. There is no radioactive peak in the phenylalanine profile over steps 1–4. The phenylalanine at position 23 drops slowly over the next few steps due to the 'lag' that has accumulated over 23 residues because at each step 1–2% of the N-terminal amino acid is not cleaved and this causes an accumulating percentage of the polypeptide to be out of phase.

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is extensive polymorphism among mice of different strains, and between wild mice and inbred mice. Wild mice from many isolated populations appear to exhibit new *H-2* haplotypes, detected serologically as having unique or private antigenic specificities[11]. Although the search for different alleles of AgB has not been so exhaustive as for the *H-2* differences, there are preliminary indications that the rat transplantation antigens may not be nearly so polymorphic as their mouse counterparts. In 37 wild rats isolated from two localities, no new public or private red blood cell specificities were detected[12].

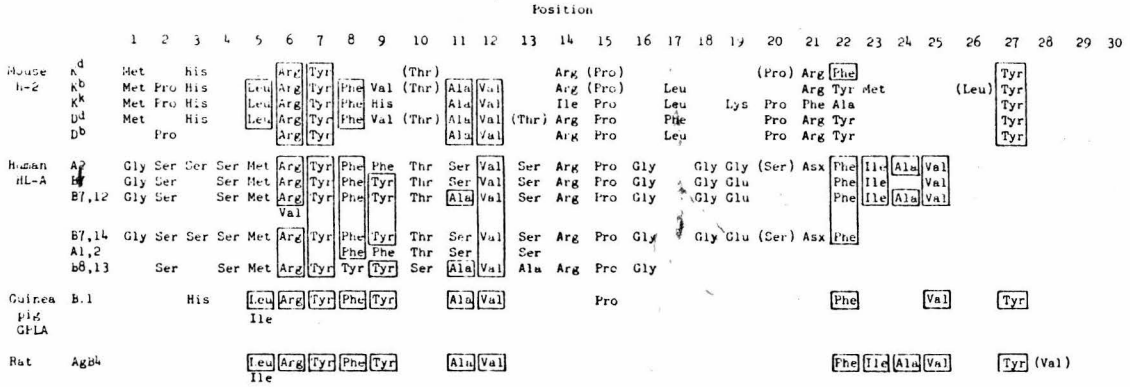
The natural function of transplantation antigens is unknown. Speculation about the function of these cell membrane-associated MHC molecules depends on whether or not extensive polymorphism is an integral aspect of the genes encoding them. Our long-term studies on rat transplantation antigens are aimed at determining if the extent of amino acid sequence variation exhibited among these gene products is quantitatively different from that observed in the mouse transplantation antigens. Study of these antigens should give us important insights into the organisation of the transplantation antigen genes. In this regard, one question we hope to answer relates to the number of different transplantation antigens expressed by the AgB complex. One additional goal in this study is to analyse the rate of evolution of this system in rodents, as amino acid sequence information is also available on the transplantation antigens from mice and guinea pigs.

AgB4 antigens were isolated from biosynthetically labelled BN.B4 spleen cells by immune precipitation. The BN.B4 strain has been made partially congeneic for the AgB4 haplotype by backcrossing (BN × DA) F1 to BN for 11 generations before breeding *inter se* to obtain homozygotes. The antiserum was prepared by skin grafting followed by immunisation of (Lewis × BN.B2) F1 rats with DA (AgB4) lymphocytes. The profile of the BN.B4 immune precipitate separated by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) is shown in Fig. 1. In addition to the 45,000 MW component, the presumptive transplantation antigens, this antiserum detects Ia-like molecules of 28,000 and 35,000 MW. The β-2-microglobulin component of 12,000 MW associated with the 45,000 MW component runs at the dye front on 10% gels. Its presence has been confirmed on 15% gels. The (Lewis × BN.B2) F1 anti-DA antiserum has potential reactivity with private specificity 4 and with the public specificity 30[8] when tested on BN.B4 spleen cell lysates. We are confident that this antiserum detects the AgB transplantation antigen for two reasons. (1) The antiserum was prepared in and tested on cells from animals essentially congeneic except for the AgB complex, thus virtually eliminating the detection of antigens coded by genes that are not closely linked to AgB. (2) The same 45,000 MW component is completely cleared from the soluble antigen preparation by precipitation with an antiserum prepared in the same strain combination against purified red blood cells, which are a rich source of transplantation antigens[8].

The 45,000 MW components were labelled in short-term tissue culture with seven tritiated amino acids, isolated from 10% SDS-polyacrylamide gels, and submitted to microsequence analysis as previously described[13]. The phenylthiohydantoin (PTH) amino acid derivatives from each cycle were separated by high pressure liquid chromatography in the presence of unlabelled PTH standards and assayed for radioactivity on a scintillation counter. Data from the rat transplantation antigens are presented in Fig. 2. The assignment of residues is based on (1) a sharp rise in radioactivity associated with a particular amino acid at a given position, followed by a more gradual decline in subsequent positions, and (2) consistency with the repetitive yield calculated where possible for each amino acid. Several important points emerge from these data. First, this was technically an excellent sequenator run with a repetitive yield of 97%. Second, a single major residue is identified at positions 6, 7, 8, 9, 11, 12, 22, 23, 24, 25, 27 and 28. Third, two residues, leucine and isoleucine, are present at position 5. Furthermore, the isoleucine at position 5 is present in about half the yield expected if a repetitive yield curve is calculated from the isoleucine at position 23 (see Fig. 2 legend). The peak height of leucine is equivalent to that of isoleucine and this suggests they are present in roughly equimolar yields because past experience has shown that both of these amino acids incorporate equally well. The conclusion to be drawn from these data is that the immune precipitate of the AgB4 transplantation antigens has two major sequences which are identical at 12 positions and differ at one position.

The partial amino acid sequence data from rat transplantation antigens are presented with data from their mouse, human and guinea pig counterparts in Fig. 3. Three points are of interest. First, the rat AgB4 sequences are clearly homologous to the MHC transplantation antigens of other mammals. For the rather small number of residues identified, the rat transplantation antigens show between 60% and 80% sequence identity with other mammalian transplantation antigens. Indeed, each residue present in the rat molecules is present in one of the other mammalian transplantation antigens. These homologies demonstrate clearly that the genes encoding the transplantation antigens from mouse, human, guinea pig and rat descended from a common ancestor. Second, the presence of leucine and isoleucine at position 5 suggests that we have isolated at least two transplantation antigens which are identical at 12 of 13 positions compared. The leucine–isoleucine polymorphism has been noted in the sequence of transplantation antigens from rats of the AgB5 haplotype (data not shown) and in the transplantation antigens from the guinea pig[14,15] (Fig. 3). Accordingly, this

Fig. 3 Amino acid sequences of major histocompatibility complex transplantation molecules. Sequences other than that of the rat were compiled by J. Silver from data presented in ref. 19. Boxes denote residues that are identical in three or more species.



polymorphism seems to be present in the transplantation antigens of several rodents. It is likely that these sequences represent two major transplantation antigens, perhaps equivalent to the *K* and *D* antigens of the mouse. Finally, it is interesting to note that there is only one residue difference in 12 (~8%) noted between the two rat transplantation antigens. After more complete sequence analysis it will be interesting to determine whether the rat transplantation antigens differ as extensively as their mouse counterparts[4].

Obviously, more data will be required before firm conclusions can be reached on the sequence diversity of individual rat transplantation antigens and on the implications this diversity has for the extent of polymorphism this system exhibits. The use of monoclonal antibodies to transplantation anitgens may allow us to separate individual gene products[16]. In addition, we hope to make use of recombinants in the AgB complex to determine whether the genes encoding the transplantation antigens are both on the same side of the existent recombinants[17]. This will be possible if the putative *K* and *D* homologues in the rat exhibit haplotype-associated sequence differences. More detailed amino acid sequence and comparative peptide map studies are in progress.

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VI. The Partial N-terminal Amino Acid Sequences of Transplantation Antigens from Three Additional Rat Strains

The major histocompatibility complex (MHC) of mammals is a complex of closely linked genes which appear to control a variety of immune phenomena. Such superficially diverse functions as control of graft rejection, level of immune responsiveness to various antigens, and serum levels of complement components are associated with genes of the MHC, for virtually all species studied (reviewed in 1). The functions of these genes in the mouse appear to be mediated for the most part by their cell surface glycoprotein products: the transplantation antigens and the immune response region-associated (Ia) antigens.

The major histocompatibility system in rats is called AgB (2), or, more recently, H-1 (3). Its identification was based on the demonstration that allelic genes controlling the production of red cell antigens were responsible for antigens determining allograft survival (4) and also for graft versus host reactions (2). The development of congenic and recombinant rat strains has not been as productive as such work with mice, and the immunogenetic studies of the rat H-1 complex, which rely on such strains, have been less extensive than those of the mouse H-2 region. At least ten distinct H-1 haplotypes have been defined in inbred rats (5). In two studies of wild rats isolated from several different locales, no new H-1 specificities were identified. All of the wild rats shared H-1 specificities with inbred strains (6, 7). The observation of such a limited number of private specificities in the rat is in stark contrast with the finding that there is extensive polymorphism of transplantation antigens in the mouse (8) and human (9). The potential for restricted polymorphism is one aspect of interest in the rat system, as it constrains the role of polymorphism in the function of the transplantation antigens.

Each H-1 haplotype is defined by a single private specificity (5), and there is no convincing evidence from intra-H-1 recombinants to indicate the presence of more than one major transplantation locus in the rat. A number of species have been shown to have at least two serologically defined transplantation antigens, including mouse, human, chimpanzee, dog, rhesus monkey, and chicken (10). As the number of separate transplantation antigens may be also relevant to their function, it would be interesting to know if two or more antigens are also present in the rat.

The major obstacle to serological characterization of the H-1 complex is the problem of developing monospecific reagents. Available alloantisera, produced in congenic combinations, potentially recognizes all MHC alloantigens. In fact, we know that the antisera used in this study contains antibodies which detect both rat transplantation antigens and a homologue of mouse and human Ia antigens. I am currently circumventing the problem of multispecific antisera by chemical characterization of the serologically detectable products of the H-1 complex. One can separate proteins of different molecular weights, and submit the isolated proteins to microsequence analysis to determine the number and nature of the antigens coded for by a given H-1 haplotype.

This chapter reports a continuation of our recent work on the transplantation antigens of the rat. I address two fundamental questions regarding the molecular complexity of these antigens: 1) What is the extent of diversity between antigens of different haplotypes, and 2) how many antigens are coded by each rat haplotype, and how different are they, if at all, from one another?

MATERIALS AND METHODS

Rats: BN and August inbred rats were provided by the Wistar Institute, Philadelphia, Pennsylvania. Lewis rats, also highly inbred, were obtained from Microbiological Associates, Bethesda, Maryland. Antisera used in this study were prepared at the Wistar Institute, and the strain combinations used to produce them are listed in Table 1.

Isolation of MHC transplantation antigens: This method has been described (11, and Chapters V and VII of this thesis). Briefly, radiolabeled rat antigens were isolated by indirect immunoprecipitation using a Staphylococcus aureus immunoadsorption procedure (12). The collected antigens were subjected to SDS-polyacrylamide gel electrophoresis, and eluted in 0.001% SDS, dialyzed, lyophilized and prepared for sequencing. Tritiated amino acids incorporated into August and Lewis rat spleen cells were alanine, tyrosine, arginine, valine, proline, phenylalanine, isoleucine, and leucine. Arginine and proline were omitted from the BN spleen cell cultures.

Microsequence analysis: Rat transplantation antigens were analyzed by microsequencing techniques as described in (11) and in Chapters V and VII of this thesis. Each rat antigen was sequenced in the presence of sperm whale apomyoglobin on a modified Beckman 890 Sequenator (13), with an automated conversion flask. Each residue was separated in the presence of cold PTH-amino acid standards on a Du Pont high pressure liquid chromatography unit, and the separated PTH-amino acids analyzed for radioactivity on a scintillation counter.

RESULTS

The isolation of H-1 transplantation antigens is based on their specific reaction with antisera produced in hybrid, and in some cases partially congenic, rats (Table 1). We have determined that these reagents are haplotype specific, but, as illustrated by the fact that two of the antisera precipitate rat Ia antigens[1], they are not restricted in their specificity for the transplantation antigens. Each antiserum does detect primarily rat MHC antigens.

H-1 antigens, obtained by indirect immunoprecipitation, were separated on one-dimensional SDS-polyacrylamide gels (as described in 14). The transplantation antigens, with a characteristic apparent molecular weight of approximately 45,000, were isolated from the gels, dialyzed to remove buffer salts and SDS, and submitted to microsequence analysis in the presence of carrier sperm whale apomyoglobin. The myoglobin sequence was used to monitor the progress of the sequencing of the radiolabeled H-1 proteins. Residues were analyzed by high pressure liquid chromatography (HPLC) in the presence of unlabeled PTH-amino acids. The PTH-amino acid peaks were collected and counted on a liquid scintillation counter. The results from the HPLC analysis for three H-1 transplantation antigens are presented in Figures 1, 2 and 3. The assignment of residues in the partial amino acid sequences derived from these data is based on 1) a sharp rise in radioactivity above background levels associated with a particular amino acid, and 2) a more gradual decline following this peak.

Several points are notable in the HPLC profiles shown in Figures 1 and 2. One of the sequences, H-1^{c}, has both leucine and isoleucine at position 5.

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[1]See Chapters VII and VIII of this thesis.

H-1^{l} and H-1^{c} have these same two residues at position 17. In addition, the repetitive yield of isoleucine at position 17 in H-1^{l} is about half of the value that would be expected from the analysis of isoleucine at position 23. Likewise, the yield of isoleucine at position 10 in H-1^{c} is less than would be predicted by the amount found at 17. All of these results indicate that there are probably two molecules being sequenced in H-1^{c} and H-1^{l} antigen preparations. Within each of these haplotypes, the only differences between sequences were those involving leucine or isoleucine. Only one sequence was seen by microsequence analysis of the BN (H-1^{n}) transplantation antigen.

There is some microheterogeneity in the H-1^{c} sequence which may be due to the presence of a minor sequence in the preparation. For example, the amount of radioactivity in leucine at position 3 is much lower than in the leucine peaks at positions 5 and 17, and it probably represents a residue from a contaminating protein.

I have made the residue assignments for the three transplantation molecules shown in Table 2, along with the N-terminal sequence of H-1^{a} (14) for comparison. It can be seen that the H-1 antigens are quite similar to one another. Four H-1 antigens share 5 of 10 residues which may be reliably compared. The three H-1 antigens studied in this set of experiments share nine of eleven residues. The residues which differ between haplotypes are at position 9 (presence or absence of tyrosine), position 22 (tyrosine or phenylalanine), positions 1 and 10 (presence or absence of isoleucine), and position 5 (presence or absence of leucine and isoleucine). Each of the four strains can be distinguished from the other three by its sequence over the N-terminal 28 residues.

There are also homologies between the set of rat H-1 molecules and the transplantation antigens of other species[2] (data not shown). Amino acids in the rat are identical at every position with at least one of the other sequences, except at position 10 (isoleucine in rats), for the 8-15 residues which can be compared.

DISCUSSION

This chapter reports a continuation of my work (14) on serologically isolated rat transplantation antigens. The H-1 transplantation antigens from three additional rat strains have been shown to be similar to but distinct from one another, while retaining close homology to the transplantation molecules of other species (14). Even the polymorphism seen in the rat antigens at position 22 (phenylalanine or tyrosine) is identical to the one seen in mouse H-2K molecules at position 22 (14). Furthermore, the presence in two rat strains of two amino acids, leucine and isoleucine, at position 5 mimics the situation in the guinea pig transplantation antigen sequence, where leucine and isoleucine are also found at position 5 (15).

It should be stressed that the antisera used for the H-1 antigen isolations has the potential to react with polymorphic antigens from the entire H-1 complex. These antisera, therefore, while haplotype-specific, are not monospecific for one antigen encoded by this region. Although there has been no previous report of multiple H-1 linked transplantation antigens in the rat (5), if two or more H-1 transplantation antigens do exist within each haplotype, the antisera we have used could, and apparently do, detect both.

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[2]For comparison, see Figure 3 in Chapter V of this thesis.

The evidence presented in this chapter, suggesting that I have in fact isolated two such transplantation antigens from each of three rat haplotypes, must be interpreted with some caution. The only differences between these molecules found within one haplotype are leucine-isoleucine substitutions, found in nearly equimolar amounts. These amino acids are found in adjacent peaks on high pressure liquid chromatography. There is a certain amount of overlap occasionally found between adjacent peaks, but, it should be pointed out, never as much as 50%. Rechromatography of PTH-isoleucine recovered from high pressure liquid chromatography of a mixture of PTH-isoleucine and PTH-leucine (data not shown) indicated that unlabeled PTH-leucine used as a standard does not contaminate the PTH-isoleucine peak more than 5%. The peaks of isoleucine at positions 5 and 17 in the H-1^a and H-1^c sequences, therefore, contain more radioactivity than would be predicted by overlap contamination from the leucine peaks found at these same positions. However, I have two reservations about the assignment of two H-1 transplantation molecules to three rat strains based on the leucine-isoleucine heterogeneity. The only difference between two molecules found within a haplotype always involves leucine and isoleucine. While it is true that the repetitive yield discrepancies indicate two molecules in these three rat strains, these also always involve isoleucine. However, it should be noted that differences between K and D molecules within a given H-2 haplotype[3] often center on whether or not leucine is present at position 5 (K^b vs D^b, K^q vs D^q), or at position 17 (K^d vs D^d, K^s vs D^s). In these four examples, the leucine polymorphism accounts for

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[3]The data for H-2K and H-2D sequences were kindly communicated to me by Dr. Richard M. Maizels (Immunogenetics, submitted).

one half (one of two polymorphic residues) of the intrastrain differences between the N-terminal sequences of K and D molecules. Therefore, the predominance of leucine in the doublets in the rat sequences is not without precedent.

The second reservation I have about the postulated existence of two transplantation antigens within one haplotype is based on a genetic argument. The reasoning is as follows: H-1^a sequences have a haplotype-associated residue (tyrosine at position 9) which is not found in three other rat strains. The repetitive yield of tyrosine in the microsequence data of H-1^a indicates that the tyrosines at position 7 and position 9 are found in equimolar quantities. Therefore, it is likely that all H-1^a molecules have tyrosines at both position 7 and at position 9. We find both leucine and isoleucine at position 5 which, most simply, would mean that the two molecules have the sequences $\overset{5\ \ 7\ \ 9}{\text{—L—Y—Y—}}$ and $\overset{5\ \ 7\ \ 9}{\text{—I—Y—Y—}}$. The existence of a haplotype-associated residue on both molecules implies that the duplication event which may have produced two transplantation antigen genes (16), and the subsequent substitution of isoleucine for leucine, occurred after the fixation of the mutation resulting in the haplotype-associated amino acid substitution. Thus, this would imply that the H-1^a duplication is a relatively recent event compared to the divergence of rat haplotypes. This scheme requires two independent but identical, or parallel, mutations, a postulate generally viewed as unattractive by those who study molecular evolution. It is difficult to compare this situation with H-2: There are no residues in mouse H-2K and H-2D sequences which are absolutely haplotype-specific. However, if one were comparing only H-2^s and H-2^q (17), it would appear that the presence of valine at position 9 was associated with H-2^s and not H-2^q. However, H-$2K^q$ and H-$2D^q$, like the H-1^a rat sequences, differ from one another at position 5 (presence or absence of leucine). These

examples serve to point out that, in two species, genetic mechanisms must exist that generate what appear to be parallel substitutions in molecules found both within and among haplotypes of major transplantation antigens.

Finally, the existence of two H-1 transplantation antigens has not yet been demonstrated in the BN rat strain. It is likely that if there are two $H\text{-}1^n$ antigens, the N-termini of both are identical to one another. It is possible, too, that one of the $H\text{-}1^n$ antigens has a blocked N-terminus, or that it is, for some reason, very poorly labeled. It is also possible that the BN rat strain expresses only one major transplantation antigen on the lymphoid cells in the spleen.

This study has posed several interesting questions about the number and diversity of rat transplantation antigens. Further chemical analysis of longer segments or internal peptides of H-1 antigens should provide some answers to these questions, and the results should shed light on the genetic organization and structural molecular complexity associated with the MHC.

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

Target Strain	Haplotype	Alloantiserum
Lewis	<u>H-1</u>^{l} (<u>AgB1</u>)	(August x BN.B2)F_1 anti-(Lewis x BN.B2)F_1
BN	<u>H-1</u>^{n} (<u>AgB3</u>)	(DA x BN.B2)F_1 anti-BN
August	<u>H-1</u>^{c} (<u>AgB5</u>)	(DA x Lewis)F_1 anti-August

Table II. Partial N-terminal amino acid sequences of four rat transplantation antigens*

	Position																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
H-1[l]	-				-		Tyr	Phe	-	Ile	Ala	Val		(Arg)			Leu Ile					Tyr	Ile	-	(Val)		Tyr	
H-1[n]	-				-		Tyr	Phe	-	Ile	Ala	+					Leu					Phe	Ile	-	+		Tyr	+
H-1[c]	Ile				Leu Ile	(Arg)	Tyr	Phe	-	Ile	Ala	Val			Pro		Leu Ile					Tyr	Ile	-	Val		Tyr	Val
H-1[a]	-				Leu Ile	Arg	Tyr	Phe	Tyr	-	Ala	Val										Phe	Ile	Ala	Val		Tyr	Val

*Dashes in sequences indicate that the amino acids seen in other rat molecules at this position are not present. Parentheses are used to indicate some uncertainty about the assignment of the amino acid. H-1[a] results are previously published ( ).

+Valine was not sufficiently labeled in the BN (H-1[n]) preparation to determine whether or not valine is present at these positions.

Figure 1. Amino acid sequence data from the August ($\underline{H\text{-}1}^{c}$) rat transplantation antigens. The amount of radioactivity associated with each of the incorporated amino acids is plotted against residue number. Repetitive yields were calculated for each amino acid where possible and are indicated by a dot-dash line. Where only one peak is seen for an amino acid, the repetitive yield indicated has been calculated from an average of the other repetitive yields found in this sequence.

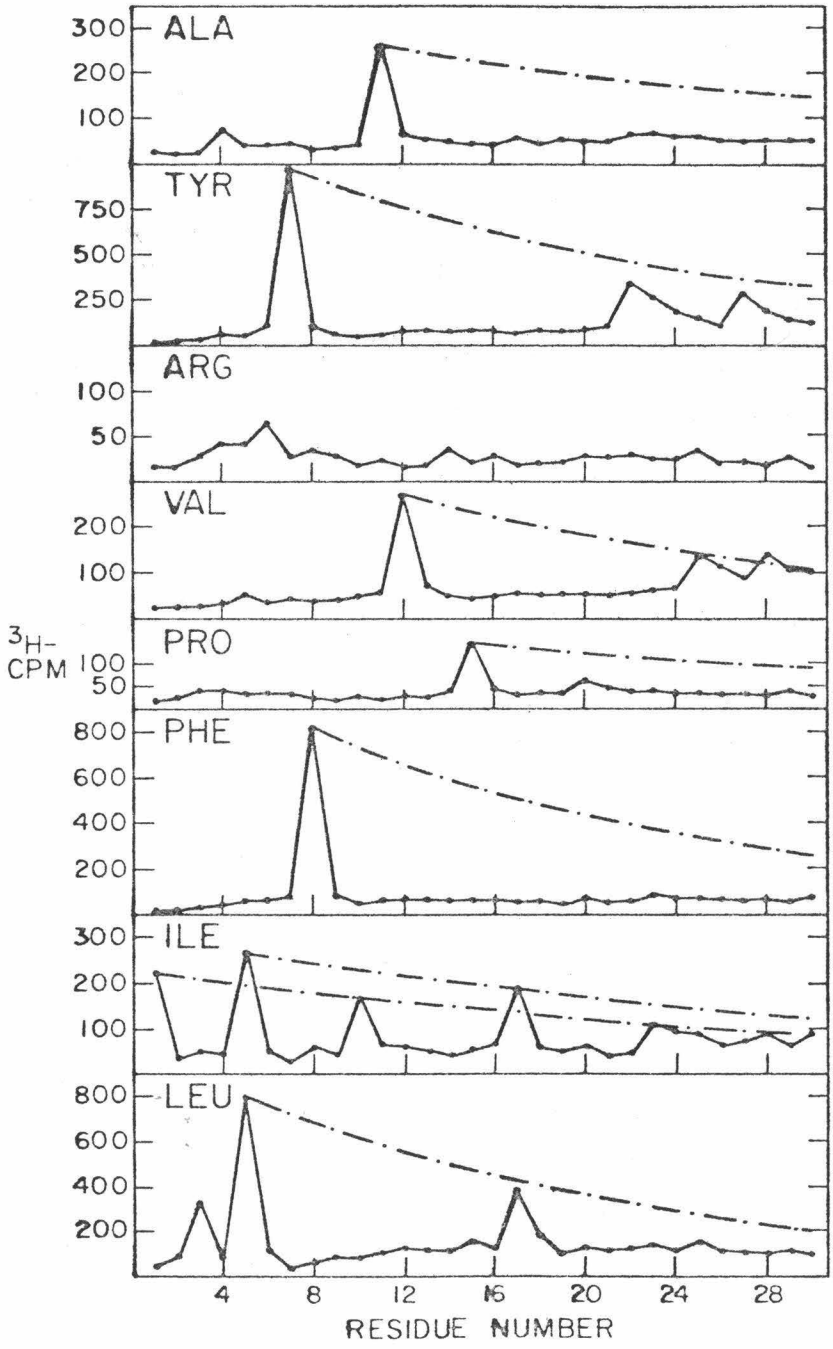

ALA
TYR
ARG
VAL
PRO
PHE
ILE
LEU
300
200
100
750
500
250
100
50
200
100
100
50
800
600
400
200
300
200
100
800
600
400
200
4
8
12
16
20
24
28
RESIDUE NUMBER
3H-
CPM

Figure 2. Amino acid sequence data from the Lewis (H-1^{l}) rat transplantation antigens. Data are presented as in Figure 1. Dashed line between positions 5 and 7 indicates that residues could not be assigned at position 6, due to a failure of the HPLC column during analysis of this step.

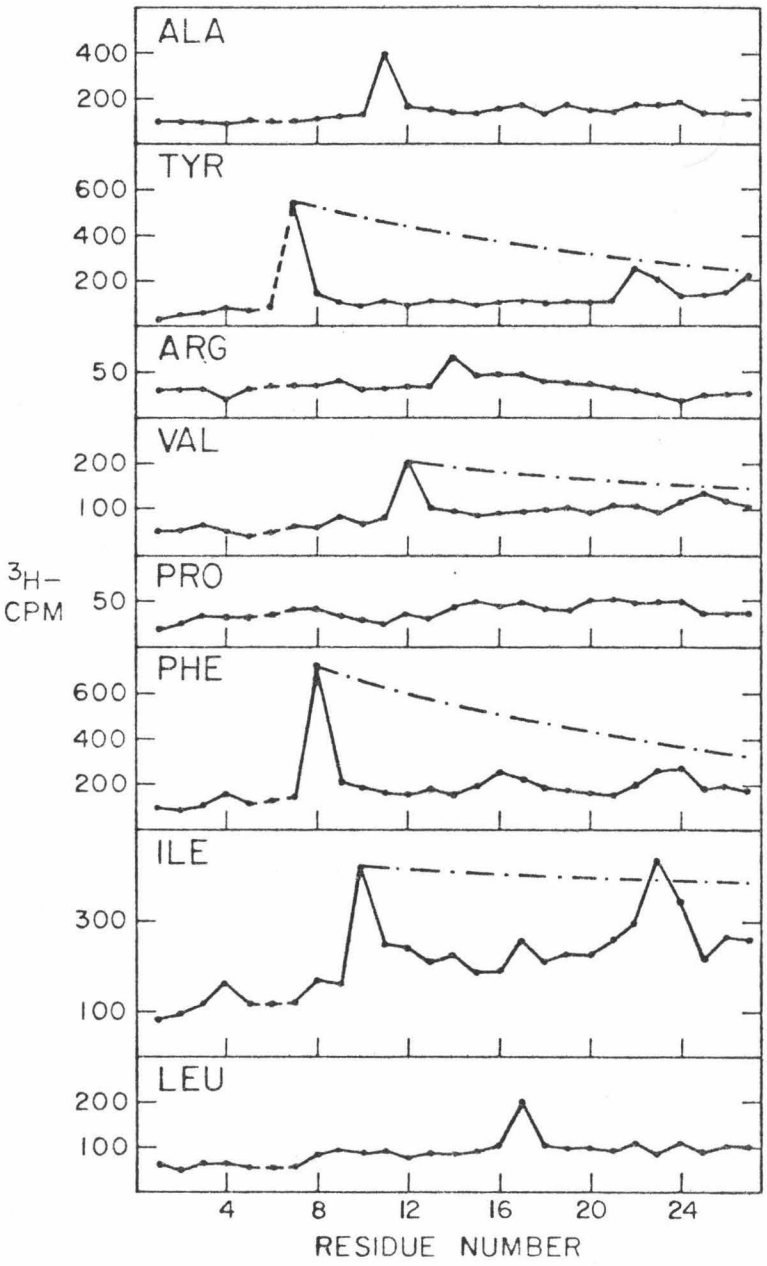
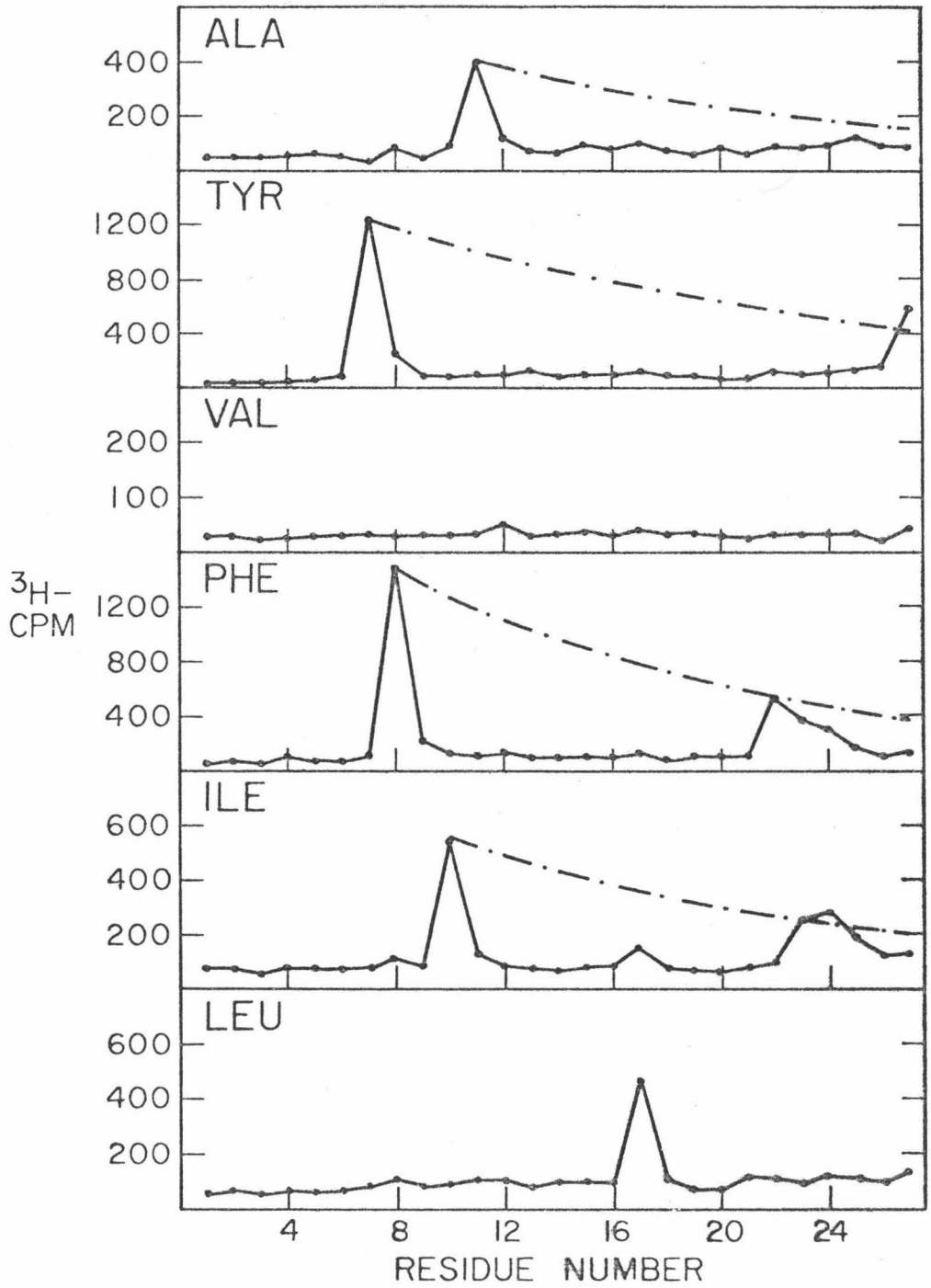

ALA
400
200
TYR
600
400
200
ARG
50
VAL
200
100
PRO
50
PHE
600
400
200
ILE
300
100
LEU
200
100
^{3}H–
CPM
4
8
12
16
20
24
RESIDUE NUMBER

Figure 3. Amino acid sequence data from the BN (H-1^n) rat transplantation antigen(s). Data are presented as in Figure 1.


ALA
400
200
TYR
1200
800
400
VAL
200
100
PHE
1200
800
400
ILE
600
400
200
LEU
600
400
200
^{3}H-
CPM
4
8
12
16
20
24
RESIDUE NUMBER

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VII. Chemical Characterization of Rat Ia Antigens

I. Microsequence Analysis of Ia Antigens from Three Rat Strains

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ABSTRACT

Rat homologues of mouse Ia antigens are identified in three rat strains by partial N-terminal amino acid sequence analysis. Rat Ia antigens were isolated by indirect immune precipitation using specific rat alloantisera. Rat Ia antigens consist of two components designated α and β on the basis of their migration on SDS-polyacrylamide gel electrophoresis. Partial microsequence analysis of each of the α components of the H-1^a, H-1^l and H-1^n haplotypes yields a single homogeneous sequence which is identical among the three haplotypes and is strikingly homologous to the mouse I-EC α polypeptides and to the human p34 Ia polypeptide. N-terminal microsequence analysis of the β components shows that a mixture of polypeptides is present for each haplotype. There are differences in these sequences among the three haplotypes. There are potential homologies between the rat β sequences and the sequences of mouse I-A and I-EC β polypeptides. The implications of these observations for the molecular organization of rat Ia antigens and for the genetic fine structure of the rat MHC are discussed.

The major histocompatibility complex (MHC) of mammals is perhaps the most studied and least understood complex of genetic information of current interest to immunogeneticists. The mouse MHC (H-2) is the best defined of these systems, owing to the existence of a large number of congenic and recombinant inbred strains. Three classes of serologically detectable products have been described which are encoded by the murine H-2 complex (reviewed in 1). The K and D major transplantation antigens are encoded by genes which are found at either end of the H-2 complex. Intercalated between the genes encoding the K and D molecules are two additional regions, I and S. The I region codes for a class of molecules which are designated the Ia (immune response region associated) antigens. The two classes of molecules are cell surface glycoproteins whose predominant expression seems to be on lymphoid cells, and in the case of the Ia antigens, expression appears to be generally restricted to these cells. The S region codes for molecules which are involved early in the complement activation pathway (2, 3, 4).

Systems other than that of the mouse are more difficult to study, primarily due to the lack of well-characterized inbred strains and the paucity of recombinant lines in which regions of the MHC are separated. In spite of this difficulty, genes for all three classes of molecules have been described (or are inferred) to be in close linkage with the MHC of several species other than the mouse, including man (5), rhesus monkey (6), guinea pig (7), and chicken (8).

The rat MHC (called H-1 or AgB) is known to be comprised of two loci which have been defined by recombinational events (9, 10, 11, 12). The H-1A locus is associated with strong graft rejection and the serologically defined transplantation antigens. The H-1B locus contains information for the mixed lymphocyte response determinants and appears to be associated with immune response genes. These

appear to be analogous to the regions encoding the K and D molecules and the Ia antigens, respectively, in the mouse. There are currently no known recombinants which further subdivide either of these two H-1 loci.

We have been involved in the chemical characterization of MHC alloantigens from mouse (13, 14, 15, 16), guinea pig (17, and McMillan et al., in preparation), and more recently, rat (18). We have approached the study of rat MHC alloantigens from the point of view that evolutionary and genetic information can be obtained in the absence of a clear understanding of the fine genetic organization and serology of the complex by examining chemical homologies with MHC products of other species. We have shown the validity of this approach in our studies of the rat major transplantation antigens (18). In this paper, we present data which suggest that multiple Ia-like molecules are encoded by the rat H-1 complex.

MATERIALS AND METHODS

Rats and alloantisera: Inbred BN and BN.B4 rats were obtained from the Wistar Institute, Philadelphia, Pennsylvania. Inbred Lewis rats were obtained from Microbiological Associates, Bethesda, Maryland. BN.B4 rats were produced by backcrossing DA (H-1 a) rats with BN rats for 11 generations, followed by breeding inter se to obtain homozygotes. We have used three rat alloantisera which were prepared and tested by Dr. Goetze at the Wistar Institute. The strain combinations used in the preparation of the specific alloantisera and the corresponding target strains are listed in Table 1.

Preparation of biosynthetically radiolabeled antigens: Single cell suspensions were prepared from the spleens of individual rats and purified on Ficoll-isopaque

gradients. Lymphocytes (10^8) were cultured for 4-5 hr in 4 ml Hanks balanced salt solution (HBSS) containing 15 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 2% fetal calf serum and 1 mCi each of tritiated alanine, tyrosine, valine, phenylalanine, isoleucine, and leucine (New England Nuclear). Tritiated proline was also included in the cultures of Lewis and BN.B4 spleen cells. The cells were washed twice in HBSS, then lysed with 2 or 4 ml of 10 mM Tris/0.15 M NaCl/0.25% NP-40 (Shell Chemical Co.), at pH 7.3, on ice for 20 min. Debris was removed by centrifugation and the supernatant containing radiolabeled antigens was stored frozen.

Isolation of alloantigens for microsequencing: Radiolabeled rat MHC antigens were isolated by indirect immune precipitation using specific alloantisera and Staphylococcus aureus (19). The resulting immunoprecipitates were purified and separated into their constituent components by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were 10% polyacrylamide and were run under reducing conditions (20). Rat Ia-like α and β polypeptides were eluted separately from the gels, mixed with 2 mg carrier ovalbumin, lyophilized, and dialyzed extensively against 0.001% SDS.

Microsequence analysis: The α and β components isolated by SDS-PAGE were submitted to automatic sequence analysis on a modified Beckman 890 A sequenator (21) using 2 mg sperm whale apomyoglobin as carrier to monitor the performance of the sequenator. At each step, the labeled phenyl-thiohydantoin (PTH) derivatives were mixed with appropriate unlabeled PTH amino acid derivatives and separated on a Du Pont 830 high pressure liquid chromatograph, using a Zorbax ODS column. The individual PTH-amino acid fractions were collected and counted for radioactivity on a Beckman LS 9000 scintillation counter.

RESULTS

Isolation and preparation of rat Ia molecules

Rat MHC antigens were isolated from biosynthetically radiolabeled spleen cells by indirect immune precipitation. The antisera and target strain combinations are listed in Table 1. Antisera were made in congenic or semi-congenic combinations and their specificities determined by cytotoxicity. For isolating rat MHC antigens, these antisera do not cross react among the strains of different haplotypes we have tested. Therefore, they detect private specificities and, at least in the case of the BN and BN.B4 target strains, should be limited to the products of the MHC or closely linked genes. We cannot determine the specificities more precisely, due to the lack of recombinant test strains. Thus, in terms of the potential reactivity of these antisera with regions of the MHC, we shall assume they detect some or all of the polymorphic products.

The use of these rat alloantisera in indirect immune precipitation of labeled BN, BN.B4, and Lewis detergent lysates yields two identifiable classes of MHC products by SDS-PAGE (Figure 1). These are typical transplantation antigens (∽45,000 mw with associated β-2 microglobulin) and Ia-like antigens (∽35,000 and 27,000 mw chains). We have identified the 45,000 mw components in each case as homologues of mouse and human transplantation antigens by micro-sequence analysis (18). The remaining components, designated α and β on the basis of their characteristic migration on SDS-PAGE, appear to be homologues of mouse Ia antigens. We will continue to refer to them as Ia antigens for the sake of simplicity, although the proper nomenclature remains to be established.

We have isolated rat Ia antigens from three strains of rats for this study: BN, BN.B4, and Lewis. The α and β polypeptides were separately sequences in the presence of 1 mg sperm whale apomyoglobin carrier. The repetitive yields for the carrier myoglobin ranged from 92% to 96% and are comparable for the radiolabeled rat Ia polypeptides. The microsequence data from the high pressure liquid chromatography analysis of the BN ($\underline{H\text{-}1}^{n}$) α and β polypeptides are shown in Figure 2.

Rat Ia α components appear to be homogeneous by microsequencing

The N-terminal sequence of the BN α component (shown in Figure 2a) and the N-terminal sequences of the Lewis and BN.B4 α components are typical of homogeneous polypeptides. Each identified residue is clearly marked by an associated rise and fall of radioactivity and conforms approximately to the calculated repetitive yield curve (dashed line). There are no unexplained minor peaks, and there is no evidence for more than one amino acid residue at any one position in the sequence. Four minor peaks are evident in Figure 2a: alanine at position 13, and phenylananine at positions 1, 7 and 8. Each of these minor peaks is due to contamination from the overlap of adjacent amino acids in the high pressure liquid chromatography separation (i.e., tyrosine contaminated alanine, and isoleucine contaminated phenylalanine). The rat Ia α sequences derived from these data are shown in Figure 3.

Rat Ia β Components are heterogeneous by microsequencing

The sequence of the BN β component (Figure 2b) appears to be heterogeneous in that the data are consistent with a mixture of at least two (and possibly more) sequences. Two features of the sequence data in Figure 2b suggest the

presence of multiple polypeptide species. 1) There appear to be two residues at the certain positions (e.g., leucine and phenylalanine at position 9, and tyrosine and phenylalanine at positions 18 and 19). These cannot be explained by overlap since, in each case, these residues are not adjacent in their elution by high pressure liquid chromatography. 2) The quantitative yields of phenylalanine and tyrosine differ from those expected for a single sequence at certain positions. For example, tyrosine at positions 12 and 17 do not conform to a single repetitive yield curve. Likewise, the yield of phenylalanine at position 7 is half that expected for a single molecule when compared to the phenylalanine at position 8. We know of no technical complexities which would explain the heterogeneity of the β chains other than that there are two or more polypeptides being sequenced. The BN β sequence has been repeated and has yielded the same result.

The sequence data for the Lewis and BN.B4 β components exhibit complexities similar to those described for the BN β component. Although these data are difficult to interpret, we have constructed a tentative sequence for the β component of each haplotype, and these are summarized in Figure 4.

Comparisons of rat Ia polypeptides

The sequences of the rat Ia α and β polypeptides derived from the Lewis (H-1^{l}), BN (H-1^{n}), and BN.B4 (H-1^{a}) rat strains are summarized in Figures 3 and 4. The sequences of the human Ia, guinea pig Ia, and mouse I-A and I-EC subregion Ia polypeptides which have been characterized are included for comparison. Identical residue assignments in these Ia polypeptides are indicated with boxes. Dashes in the sequences indicate that residues identified in another rat or mouse sequence are absent. For example, isoleucine at position 4 of the mouse I-A sub-

region α polypeptides is not present at position 4 of the mouse I-EC α polypeptides or the rat α polypeptides. Glutamic acid, which has been identified in the human p34, was not included in our microsequence analysis. Glutamic acid, therefore, may be present at position 4 in the I-EC and rat α polypeptides.

In the rat β sequence(s) (Figure 4), there are several similarities with the mouse Ia β polypeptides at positions 8, 9, 18 and 19. The homology relationships which are evident in Figures 3 and 4 will be described fully in the Discussion.

DISCUSSION

From the results obtained by microsequence analysis, we have been able to make three general observations regarding the number, the homology relationships, and the diversity of both rat Ia α and β polypeptides. The rat α chain sequences are typical of homogeneous preparations. There is no evidence of minor sequences or alternative residues at any position where assignments were made. There are no apparent sequence differences in the Ia α components among the $H\text{-}1^n$, $H\text{-}1^l$, and $H\text{-}1^a$ haplotypes. Each of the three rat α polypeptide chains show striking homology to the mouse I-EC α chains and to the human p34 polypeptide. There is identity at 7 of 7 positions which can be compared among these α polypeptides. This suggests that α chains of Ia molecules from man, mouse, rat, and guinea pig descended from a common ancestral gene.

In contrast, the β components of the rat Ia antigen is very clearly a mixture of two or more polypeptides. The data shown in Figure 2b seem to indicate that at least two similar but not identical sequences are present. The rat β components are not identical among the three haplotypes. There

are differences in the sequences of one (or more) of the polypeptides of each haplotype at positions 1, 9, 10 and 12.

Several potential homologies among β polypeptides are evident in the sequence summaries listed in Figure 4. Some rat β chains have residues which are identical to those found in the mouse I-EC β chains (positions 1, 8, 9, 18 and 19). Similarly, there are potential homologies with the mouse I-A β chains, at positions 7, 8, 9, 11, 16 and 17. The β chains of the rat thus appear to fall into at least two groups of sequences which are homologous to those of the mouse I-A and I-EC β polypeptides. This permits us to tentatively conclude that the rat has genes encoding both A-like and EC-like β molecules in the absence of direct genetic data for the existence of these two subregions. We have serological data (presented in the following paper) which suggest that our interpretation of these sequences for the BN Ia β chains as homologues of the mouse I-A and I-EC β polypeptides, is quite reasonable. In addition, there are also identities between rat and human and guinea pig Ia β molecules (at positions 4, 7, 9, 11, 16 and 17).

In addition to residue homologies between the rat β polypeptides and the mouse I-A and I-EC subregion β polypeptides, there are residues which are not represented in any of the other β polypeptides listed in Figure 4. At this level of analysis we cannot distinguish between two possible explanations: 1) polypeptides other than the I-A and I-EC β homologues are present, or 2) the rat β polypeptides differ at these positions from their mouse homologues. It is also not clear whether each polypeptide in these preparations is recognized by specific alloantibody, or whether some are present as components of a molecular complex in which only one subunit carries the antigenic specificities.

In light of the heterogeneity observed in the rat β components by microsequence analysis, it is very curious that the rat α components yield what appears to be a single sequence. Presumably if these techniques were applied to the analysis of mouse Ia antigens using antibodies to the whole mouse MHC, both I-A and I-EC subregion polypeptides would be detected. In the rat, we can identify three polypeptides with apparent sequence homology to murine Ia antigens. These correspond to a complete murine I-EC molecule (α and β) and the β component of an I-A molecule. Our data do not rule out the possibility that a homologue of the murine I-A subregion α polypeptide exists in the rat. It may have a blocked N-terminus, in which case it would not be detected by microsequence analysis.

It is interesting to consider the consequences if an I-A α homologue is absent in the rat. First, with regard to the involvement of the two chain structure of the Ia antigens in their function (which is not known), the I-A-like α may have a role which can be equally served by an I-EC-like α chain or by another molecule. The reassortments of I-A and I-EC α chains between different β chains, however, has not been observed in the mouse (P. Jones, personal communication). Second, the lack of an I-A-like α chain may indicate that there is an interesting difference in the genetic organizations of rat and mouse MHCs. We have suggested that, in the mouse, both the α and β polypeptides of I-A subregion Ia antigens are genetically encoded within the H-2 complex (22). In the rat H-1 complex, the gene(s) which controls the expression of an I-A-like α polypeptide may be absent.

Whether or not the rat eventually reveals a full complement of Ia polypeptides which can be isolated serologically, the data presented here do suggest

that the rat I-region contains genetic information encoding at least two molecules. The following paper suggests strategies which may facilitate the molecular characterization of rat MHC products on a more sophisticated level.

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

Target Strain	Haplotype	Alloantiserum
Lewis	H-1^{l} (AgB1)	(August x BN.B2)F_1 anti-(Lewis x BN.B2)F_1
BN	H-1^{n} (AgB3)	(DA x BN.B2)F_1 anti-BN
BN.B4	H-1^{a} (AgB4)	(Lewis x BN.B2)F_1 anti-DA

Figure 1: SDS-polyacrylamide (10%) gel electrophoresis patterns of rat MHC alloantigens. Migration distance in the gel is plotted on the abscissa and radioactivity of tritiated amino acid incorporated into protein is plotted on the ordinate. The major transplantation antigen (H-1) migrates with an apparent molecular weight of 45-50,000, and the Ia α and β components have apparent molecular weights of approximately 31-35,000 and 26-29.,000, respectively.

Upper Panel: ^{3}H-phenylalanine and ^{3}H-isoleucine labeled Lewis antigens precipitated with (DA x BN.B2)F_1 anti-BN antiserum.

Center Panel: ^{3}H-tyrosine labeled BN antigens precipitated with (DA x BN.B2)F_1 anti-BN antiserum.

Lower Panel: ^{3}H-tyrosine labeled BN.B4 antigens precipitated with (Lewis x BN.B2)F_1 anti-DA antiserum.

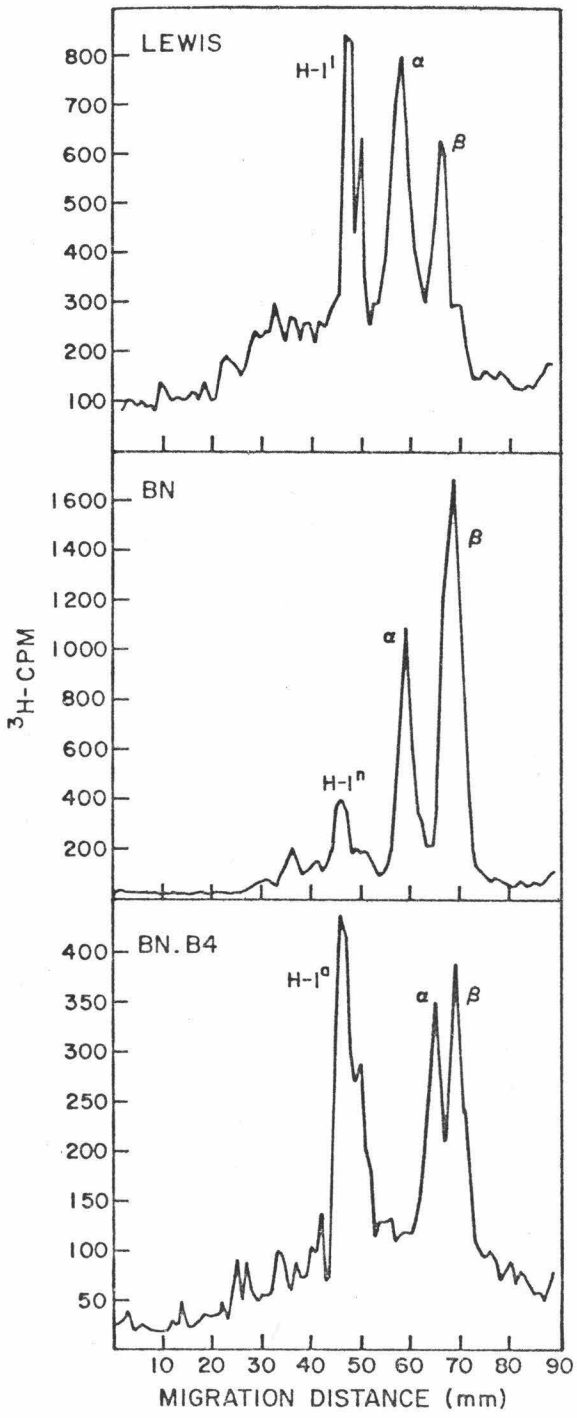

LEWIS
H-1^l
α
β
800
700
600
500
400
300
200
100
BN
H-1^n
α
β
1600
1400
1200
1000
800
600
400
200
BN.B4
H-1^a
α
β
400
350
300
250
200
150
100
50
^{3}H-CPM
10 20 30 40 50 60 70 80 90
MIGRATION DISTANCE (mm)

Figure 2: Amino acid sequence data for the BN α (Panel A) and β (panel B) components. Residue number is plotted on the abscissa and radioactivity of individual tritiated PTH-amino acids is plotted on the ordinate. --- represents the theoretical yield (cpm) for a particular amino acid at each position calculated using a repetitive yield of 95% in each panel. The repetitive yield curve is associated with the first predominant peak observed for a particular amino acid. We have assigned residues according to the following criteria: i) a sharp rise in radioactivity at one position followed by ii) a more gradual decline at positions immediately following at subsequent positions (or "lag"), and iii) approximate consistency with the repetitive yield curve.

The sequence of the BN β component (2B) indicates that a mixture of polypeptides is being sequenced as described in the text. The two repetitive yield curves in the tyrosine and phenylalanine panels are intended to indicate this heterogeneity. In our interpretation of these data, we have adhered to the criteria outlined above. Tyrosine was not assigned at position 20, nor phenylalanine at position 12, even though these points do fall approximately on the repetitive yield curves. The relatively large amounts of radioactivity at these two positions, we feel, is probably due to accumulated lag. The valine peak at position 19 was not found when this sequence was repeated, and is therefore not assigned. The dashed segment in the leucine panel indicates that the leucine sample at position 15 was lost in this sequence. In the second BN β sequence, no leucine radioactivity was found at position 15.

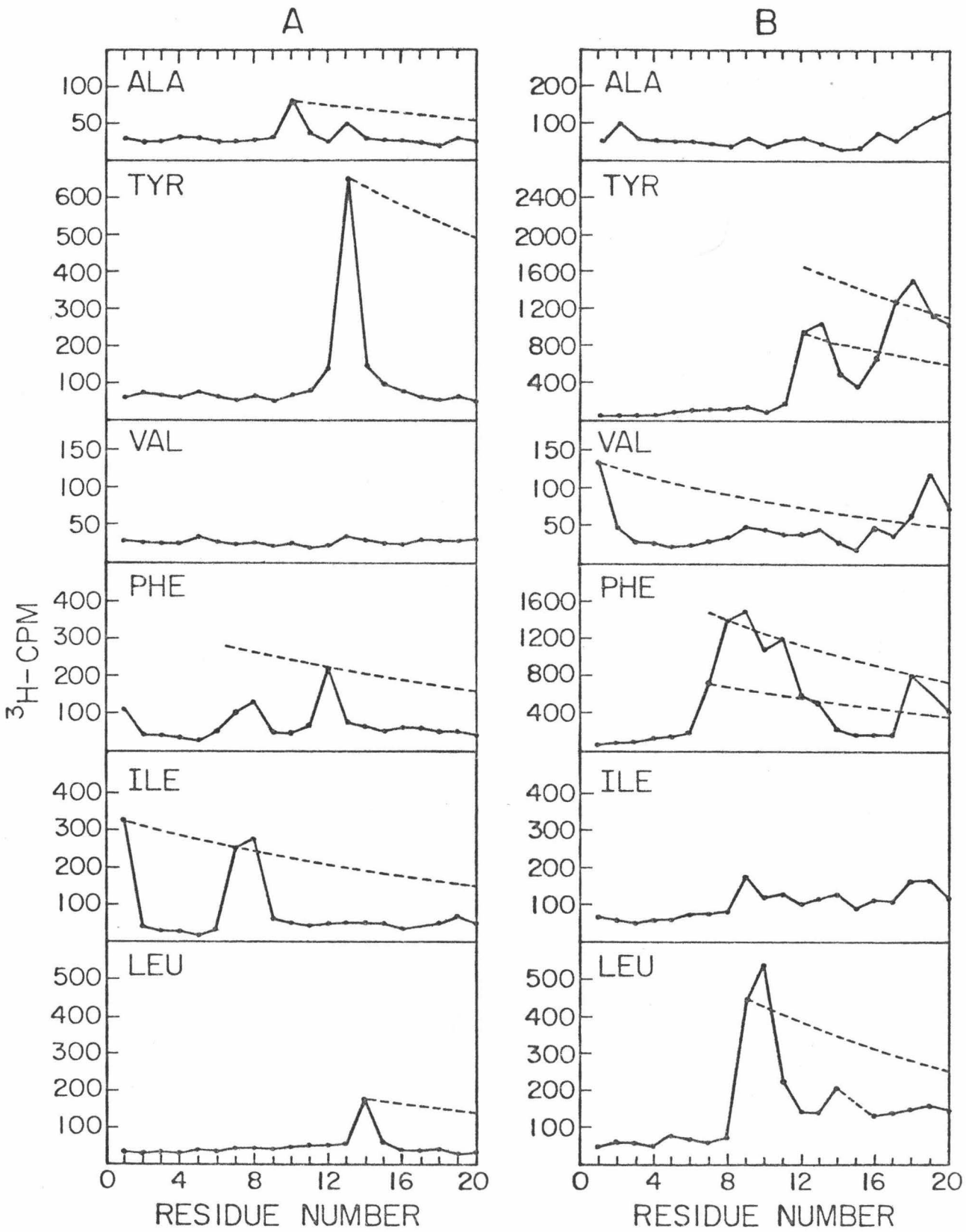

A
B
ALA
TYR
VAL
PHE
ILE
LEU
^{3}H-CPM
RESIDUE NUMBER
RESIDUE NUMBER

Figure 3: Comparisons of the partial N-terminal amino acid sequences of Ia α polypeptides. Sequence data for the N-terminal 20 residues of rat, mouse (22, and human (23) Ia α polypeptides are summarized. Dashes indicate the absence of an amino acid present at that position in another rat or mouse sequence. Identical residues are enclosed in boxes. Parentheses indicate some reservation in the assignment.

Amino acid sequences of I region alpha molecules

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Rat	H-1^{l}	Ile			-		-	Ile	Ile		Ala		Phe	Tyr							
	H-1^{a}	Ile			-		-	Ile	Ile		Ala		Phe	Tyr	Leu						
	H-1^{n}	Ile			-		-	Ile	Ile		Ala		Phe	Tyr	Leu						
	I-A^{s}	-			Ile		Ala	-	-	Val	-	Val	Tyr	-			Val	Tyr			
Mouse	I-A^{d}	-			Ile		Ala	-	-	Val	-	Phe	Tyr	-			Val	Tyr			
	I-A^{b}	-			Ile		Ala	-		Val	-		Tyr	-			Val	Tyr			
	I-A^{k}	-			Ile		Ala	-		(Val)	-		Tyr	-			Val	Tyr			
	I-EC^{k}	Ile			-		-	Ile	Ile	-	Ala		Phe	Tyr	Leu	Leu		-			
	I-EC^{d}	Ile			-		-	Ile	Ile	-	Ala		Phe	Tyr	Leu	Leu		-			
Human	p34	Ile	Lys	Glu	Glu	(Arg)	Val	Ile	Ile/Leu	Gln	Ala	Glu	Phe	Tyr	Leu	Asn	Tyr	Asp	Phe	Gln	Gly

Figure 4: Comparisons of the partial N-terminal amino acid sequences of Ia β polypeptides. Sequence data for the N-terminal 20 residues of rat, mouse (22), human (23), and guinea pig (17) Ia β polypeptides are summarized. The sequences are presented as explained in the legend to Figure 1. At positions 8 and 9, different amino acids are included in boxes. At position 8, phenylalanine in the rat β polypeptides is identical to the phenylalanine at position 8 in the mouse I-EC β polypeptides, while the valine residues in the $H\text{-}1^{l}$ and $H\text{-}1^{a}$ sequences are identical to the valine at position 8 in the mouse I-A β polypeptides. Similarly, at position 9 there are three sets of homology relationships indicated by the boxes.

Amino acid sequences of I region beta molecules

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Rat	H-1[l]	-			Pro			Phe	Val Phe	Tyr	Tyr	Phe	-								
	H-1[a]	Val			Pro			Phe	Val Phe	Tyr		(Phe)	-				(Tyr)	Tyr	(Phe)		
	H-1[n]	Val						Phe	Phe	Leu Phe	Leu	Phe	Tyr				(Tyr)	Tyr	Phe (Tyr)	(Phe) (Tyr)	
Mouse	I-A[s]	-						Phe	Val	Phe		Phe	-				Tyr	Phe	-	-	
	I-A[d]	-						Phe	Val	Val		Phe	-				Tyr	Tyr	-	-	
	I-A[b]	-				Arg			Val	Tyr							Tyr			-	
	I-A[k]	-				(Arg)			Val								Tyr			-	
	I-EC[k]	Val						-	Phe	(Leu)		Tyr	-				-	-	Phe	Tyr	
	I-EC[d]	Val					Pro	-	Phe	Leu		Tyr	Val			(Leu)	-	-	Phe	Tyr	
Human	p29	Gly	Asp	Thr	Pro	Glu		Phe	Leu	Glu	Gln	Val									
Guinea Pig	Ia[4,5]	Ile	Tyr		Pro			Phe	Leu	Phe		Phe						Tyr			
	Ia[3,5]			Leu	Pro	Arg	Arg	Phe		Phe		Phe	Lys		Tyr		Tyr				

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VIII. Chemical Characterization of Rat Ia Antigens

II. Detection of Two Ia Antigens Using Cross-Reactive Mouse Alloantisera

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SUMMARY

Ia antigens in both rats and mice are composed of two polypeptide chains, designated alpha and beta. In these experiments, rat Ia beta chains are shown by two-dimensional gel electrophoresis to have multiple components, or spots, on the autoradiograms of these gels. One subset of beta spots can be precipitated with highly specific mouse anti-I-A^k antiserum, and a different set of spots can be detected using mouse alloantiserum to the I-EC^k subregion. Both I-A-like and I-EC-like sets of beta spots are precipitated by congenic rat alloantiserum to antigens of the major histocompatibility complex (MHC). These results demonstrate for the first time that two sets of Ia antigens are present in the rat. Thus, the rat, like the mouse, may have Ia antigens encoded by at least two subregions of the rat MHC. The existence of multiple rat Ia gene products is revealed by these techniques even in the absence of formal genetic evidence of more than one Ia subregion in the rat.

The major histocompatibility complex (MHC) of mammals is a genetic region which controls a variety of immunologically significant phenomena. The information for at least one, and often two or more serologically detectable transplantation antigens is located within the MHCs of several species. The human and mouse MHCs also contain structural or regulatory genes for controlling the immune response (Ir genes), and the associated alloantigens which map to the Ir region (Ia antigens) (1).

In the rat, the 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 antibody production, skin graft rejection, graft versus host reactions, and mixed lymphocyte interactions occurring between experimentally manipulated inbred rats. As recently as 1973 (4), there was no evidence of genetic division at all within the rat MHC. The assignment of genes controlling the level of the immune response to branched synthetic polypeptides (Ir genes) was made to H-1 in 1974 (5). In 1977, evidence of recombination between the genes controlling mixed lymphocyte interactions (MLI) and the genes coding for the classical, serologically-detected (SD) red cell antigens was presented (6). Since then, there have been several studies which have also indicated that Ir (and MLI) genes are separable from SD antigens in the rat MHC (7, 8, 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 rat strains has greatly facilitated the study of the H-1 complex, but there has been no further subdivision of this region.

In recent years, chemical approaches to the identification of MHC products have been developed. Two methods commonly employed are microsequencing (10)

and acrylamide gel electrophoresis of proteins isolated from lymphoid cell detergent lysates, including both one-dimensional (11) and two-dimensional analysis (12). Most of the studies employing these techniques have relied on the isolation of MHC products by antiserum, usually prepared to antigens of well-characterized portions of the MHC. This has been very productive in the characterization of mouse MHC (H-2) antigens, where years of genetic research have provided a genetic map of H-2 with at least eight defined loci (13). This strategy has not been as productive in the rat, because antisera that are operationally monospecific have not been available for the subregions of H-1 that one would expect, by homology with the mouse MHC, to be present.

Because the rat has been extensively studied in experiments designed to reveal the role of the immune system as it functions in graft survival and rejection, we felt it was desirable to apply our efforts toward chemical characterization of molecules associated with immune functioning in this species. We are also interested in knowing whether the rat MHC is as complicated as H-2, or if the rat employs different, perhaps simpler, genetic strategies.

We know from previous studies that rat Ia-like antigens are obtainable from lymphoid cell detergent lysates by precipitation with rat alloantiserum (14, 15). Our own early work with cross-reactive mouse alloantisera, and that of Sachs et al. (16) showed that mouse antiserum can also be used to precipitate rat antigens. We considered the possibility that the highly specific mouse reagents would help us dissect classes of different rat Ia molecules. The work reported in this paper includes experiments designed to study the molecular complexity of rat Ia antigens isolated serologically with both rat and mouse alloantisera.

MATERIALS AND METHODS

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

Antisera: Antisera used in this study are listed in Tables I and II. 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 NIH, Bethesda, Maryland. All rat antisera were prepared at the Wistar Institute, Philadelphia.

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 (FCS) and 20 mM Hepes buffer. The lymphoid cells were then isolated on Ficoll-isopaque gradients (17), washed, and counted. Approximately 2×10^7 live cells were incubated at $37^{\circ}C$ for five hours in 1 ml of Hepes-HBSS-2% FCS containing 1 mCi of the ^3H-amino acid (New England Nuclear) to be assayed. Cells were washed in ice-cold medium three times after this short-term culture and lysed in ice-cold 0.01 M Tris/0.15 M NaCl/0.25% NP-40 buffer for 20 minutes. 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.

Analysis of immune precipitates: Radiolabeled antigens were precleared with fixed Staphylococcus aureus Cowan I (SaCI) (18) 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 minutes 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 minutes in 4% SDS-6 M urea. The eluates were collected by centrifugation, diluted with glycerol and buffer salts, reduced in 2% 2-mercaptoethanol, and analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (19). Gels were sliced and eluted, and the radioactivity counted in a liquid scintillation counter.

Two-dimensional gel electrophoresis: Antigens were precipiated and isolated as above, except that the complexes were eluted from the SaCI with isoelectric focusing buffer (20). Samples were then applied to non-equilibrium pH gradient gel electrophoresis exactly as described (20). The first dimensional gels were then 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 (21) and prepared for subsequent fluorography (22). Kodak XR-5 X-ray film was flashed with a strobe light before applying it to the dried gels. The autoradiograms in this paper reflect an exposure period of approximately 25 days for gels containing labeled proteins from the 3-5 x 10^6 cells originally immunoprecipitated.

RESULTS

Results with rat alloantiserum

Precipitation of labeled Lewis rat spleen cell lysates by anti-H-1^l serum gives the SDS-PAGE pattern illustrated in Figure 1. It can be seen from this gel pattern that the transplantation antigens are separated from the Ia antigens by this procedure. The larger molecular weight peak contains the H-1 transplantation molecules, and the two smaller peaks, designated alpha and beta, comprise the Ia antigens (14, 15). The transplantation antigens and the Ia antigens are very similar in their molecular weights to the MHC proteins isolated from mouse spleen cells (23). Essentially the same gel patterns are found for H-1 complex antigens from four rat strains (BN, BN.B4, BN.B2, and Lewis).

Results with mouse alloantisera

The MHC antigens from four rat haplotypes have been determined to react with highly specific alloantisera prepared against mouse Ia antigens. The results for the precipitation of Lewis (H-1^l) antigens by mouse anti-I^k antiserum are presented in Figure 2. Ia antigens from Lewis, BN.B2, BN, and BN.B4 rats can be precipitated with antiserum directed to the whole mouse I region, or to the I-A or I-EC subregions. Both α and β components of rat Ia antigens are found in the mouse antiserum immune precipitates.

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. The mouse reagent could detect no BN proteins after the antigen preparation had been cleared with (DAxBN.B2)F_1 anti BN antiserum

(Figure 3C). This indicates that the anti-$\underline{I}^k$ reagent does not recognize any Ia molecules that are not detected by the congenic rat alloantiserum.

We did reciprocal clearing experiments with more restricted mouse anti-I-region sera. Aliquots of BN spleen cell preparations were precipitated with either anti-$\underline{I\text{-}AB}^k$ or anti-$\underline{I\text{-}EC}^k$ (see Table II) serum. The supernatants of each reaction were checked for residual activity with these reagents until all precipitable material was cleared from the antigen preparations (Figure 4). These cleared supernatants were then treated with the opposite reagent, and the immune precipitates were run on gels. Anti-$\underline{I\text{-}AB}^k$ serum recognizes Ia molecules that are left behind by anti-$\underline{I\text{-}EC}^k$ serum, and vice versa. In the rat, therefore, the anti-$\underline{I\text{-}A}^k$ and anti-$\underline{I\text{-}EC}^k$ antisera react with different molecules.

Similar experiments were performed using antisera directed to the same I subregion (I-A) from two different H-2 haplotypes of mice. BN spleen cell antigens were exhaustively precipitated with an anti-$(K)^b$ $\underline{I\text{-}AB}^b$ or an anti-$\underline{I\text{-}AB}^k$ antiserum. When the supernatants of these reactions were cleared of reactivity with the homologous reagent, they were treated with the reciprocal antiserum. In this case (data not shown), no Ia antigens were recognized by anti-$\underline{I\text{-}AB}^k$ serum that were not detected by anti-$\underline{I\text{-}AB}^b$ serum and vice versa. Thus, specificities cross-reacting with antibodies to different mouse I-A subregion haplotypes reside on the same Ia molecule in the rat.

In concert with these observations on one-dimensional SDS-PAGE, we have observed that the species of Ia beta polypeptides recognized and precipitated by the rat antiserum may be subdivided into I-A-like and I-EC-like classes by virtue of their behavior of two-dimensional gels. BN rat cell preparations were precipitated with rat alloantiserum, or anti-$\underline{I\text{-}AB}^k$ serum, or anti-$\underline{I\text{-}EC}^k$ serum.

These immune precipitates were submitted to two-dimensional gel electrophoresis (Figure 5). The pattern of spots associated with the beta components isolated by the anti-I-A^k reagent, when combined with the pattern detected by the anti-I-EC^k reagent, will reconstruct the pattern of spots found in the precipitate of the rat alloantiserum. We are thus able to dissect the Ia beta chains brought down by the rat antiserum into two distinct groups as determined by their cross-reactivity with monospecific reagents prepared in the mouse: the I-A cross-reacting beta polypeptides, and the I-EC cross-reacting beta polypeptides. In comparing the pattern of spots of the alpha components, no clear differences were evident in the precipitates of BN antigens with either of the mouse antisera or with the rat antiserum. There are two reservations we have in interpreting the results with alpha polypeptide chains. The first is that ^{3}H-tyrosine, which was used to label the rat antigens in Figure 5, does not label the alpha chains very well, and the alpha chain spots are relatively faint on the autoradiographs. Second, the alpha chain region in the anti-I-A gel was technically distorted (Figure 5A). For these reasons, we cannot eliminate the possibility that heterogeneity exists between groups of alpha polypeptides detected by either of the two mouse alloantisera.

DISCUSSION

The prevalent models for the organization of the major histocompatibility complexes of various species rely heavily on similarity to, or dissimilarity from, the mouse H-2 system (1, 24). 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. We would like to know more about the possible correlation of the Ir functions with the production of alloantigenic molecules by the rat I region. The direction

of our efforts in this work has been to determine the number of antigens with genetic information in the rat I region, and assess their chemical characteristics. We define our rat Ia antigens on the basis of four criteria: their detection by rat alloantisera specifically prepared to the MHC region, their gross chemical properties of molecular weight and isoelectric focusing characteristics, their close sequence homology to the Ia antigens of other species (25), and their cross-reactions with highly specific mouse alloantisera to Ia.

We have isolated rat Ia antigens from biosynthetically labeled spleen cell detergent lysates using rat alloantisera with potential reactivity for the whole rat MHC. The rat Ia antigens are structurally similar to those of the mouse. They are composed of two polypeptide chains, alpha and beta, which have molecular weights of approximately 28,000 and 33,000, like the mouse. Rat Ia antigens are also precipitated by mouse antiserum to mouse Ia antigens. We can demonstrate at least two distinct groups of Ia antigens from the rat using two specific mouse reagents. The primary, and perhaps the only, distinction between the two groups of rat Ia antigens resides in the beta polypeptide components, where clear differences in electrophoretic behavior were observed on two-dimensional gels.

The existence of cross-reactions between rat and mouse membrane antigens is not unexpected. 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 (26). Sachs and his co-workers have presented evidence of Ia specificities shared by rats and mice, using mouse anti-rat serum and congenic mouse alloantiserum to the whole H-2 region (16). The reactivities of the mouse anti-rat lymphocyte serum in this study indicated that the mouse

cross-reactive antigen is encoded by genes located in the I-A subregion. Because all rat strains tested showed cross-reactivity with this antiserum, the genetic map location of the cross-reactive molecule in the rat could not be ascertained. The results of Sachs et al. are similar to our own in that all of the mouse antisera which react with Ia antigens from one rat strain also react with all of the haplotypes of rats available to us. It has not been demonstrated that all of the rat strains will prove to have the same two distinct and separable reactivities determined with the I-A antiserum or the I-EC antiserum. A positive difference between rat haplotypes in cross-reactivity could be of use in detecting recombination within the rat H-1 region.

The cross reactions observed seem likely to involve similar but not identical determinants on the rat Ia antigens. There are several reasons to suspect that this is the case. The mouse alloantisera are highly specific in their reactivities when tested against mouse antigens or whole cells. They discriminate both haplotype and subregion. No such discrimination with regard to haplotype is observed when they are tested on rat antigens. The relative avidity of the mouse antibodies appears to be lower when tested against rat preparations as compared to mouse antigens. Rat Ia antigens cannot be completely removed by mouse anti-I^k antisera in repeated applications, yet the Ia antigens appear to be the same in their two-dimensional gel patterns in both mouse and rat precipitations. The mouse antisera do work well on homologous antigen preparations. It is estimated that rats and mice diverged ten million years ago, which should be adequate time for the evolution of differences in the intricate structure of alloantigenic sites which nevertheless remain sufficiently similar to retain cross-reactivity. In light of this time for diversity to occur, it seems unlikely that sets of antigenic determinants which

are polymorphic within a species should be conserved between members of two different species. Various genetic models have been discussed in an attempt to explain this surprising observation (16, 27). Finally, it is also possible that antibodies exist in the mouse alloantisera which are not detected when tested against mouse cells, but which do react with rat Ia antigens bearing determinants identical to those of the immunizing antigen.

It is clear that cross-reactive determinants for I-A-like and I-EC-like molecules reside on different beta polypeptides in the BN rat strain. This provides us with evidence for two separate I region products in advance of any genetic evidence for the subregion nature of the H-1 I region. It is not clear at this time that the existence of the two molecular species necessarily implies that there are two genetic subregions in the rat I region. The possibility exists, for example, that one of the two molecules is encoded by genes outside the MHC. This is made less likely by the observation that congenic rat alloantiserum, directed only to the products of the MHC, is able to clear the antigen preparation of any molecules reactive with either mouse reagent. Interstrain diversity between beta molecules, as reflected by possible haplotype-associated residues in the microsequences of rat beta chains (25), and by the two-dimensional gel differences we find between beta chains from congenic strains (data not shown), have been understood to mean that the beta molecules are probably encoded by the MHC (28). The alpha molecules of each rat haplotype are poorly resolved by the two-dimensional gels, and we do not know whether there are differences between haplotypes in the rat Ia alpha chains. There is no evidence of interstrain diversity in the rat Ia alpha polypeptides by microsequencing (25).

Definition of the genetic organization responsible for the production of two Ia antigens in the rat will require that we characterize the cross-reactive molecules in other rat strains. We also hope to investigate the nature of the rat alpha chains. There is always a small but distinct alpha peak in the one-dimensional gel patterns of rat Ia antigens precipitated by either rat or mouse reagents. Because of the ambiguities reported in the Results section, we cannot determine from these data whether there are two distinct sets of alpha chains associated with either the I-A-like or I-EC-like beta chains. For example, it is possible that only three polypeptides are coded for by the rat I region: an I-EC alpha chain, an I-EC beta chain, and an I-A beta chain, and that the complete Ia antigen is comprised of one alpha and either beta chain. However, in mouse spleen cell Ia molecules, there is no heterogeneous mixing of the I-EC alpha chains with I-A beta chains. Further biochemical analysis should resolve the question of what restrictions are placed on the association of chains during the assembly of rat Ia antigens. We have demonstrated, for the first time, the existence of multiple 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.

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

Serum	Recipient Strain	Donor Strain	H-1 Region Detected
R150	(DA x BN.B2)F_1	BN	H-1^n
R169	(LEW.BN x BN.B2)F_1	Lewis	H-1^1
R143	(Lewis x BN.B2)F_1	DA	H-1^a
R140	(BN.B4 x BN)F_1	BN.B2	H-1^w

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

Serum	Recipient Strain	Donor Strain	Mouse I Regions Detected
031	(B10.T(6R) x B10.D2)F_1	B10.AQR	$\underline{AB}^k\underline{JE}^k$
064	(B10.S(7R) x A.CA)F_1	B10.HTT	$\underline{EC}^k$
D3b	(C3H.H-2^o x 129)F_1	C3H	$\underline{ABJEC}^k$
12	(A.TH x B10.HTT)F_1	A.TL	$\underline{AB}^k(\underline{J}^k)$
D33	(B10.D2 x A)F_1	B10.A(5R)	$\underline{AB}^b$

Figure 1. SDS-polyacrylamide gel profile of ^{3}H-phenylalanine and ^{3}H-isoleucine labeled Lewis rat antigens immunoprecipitated with rat alloantiserum to H-1[1] antigens (R169). Major transplantation antigens are in the peak labeled AgB, and the Ia peaks are designated α and β. The immune precipitate of antigens from 1×10^{6} spleen cells was applied to the gel.

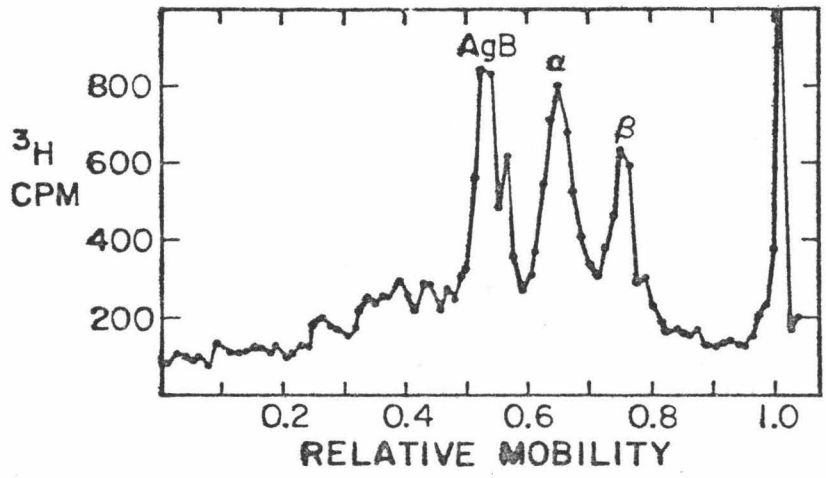

AgB
α
β
800
600
400
200
^{3}H CPM
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 2. SDS-polyacrylamide gel profile of ^{3}H-phenylalanine and ^{3}H-isoleucine labeled Lewis rat antigens immunoprecipitated with mouse alloantiserum directed to the whole mouse $\underline{I}^{k}$ subregion (031). Ia antigens are designated α and β, and the peaks designated NS contain non-specific proteins, which are also precipitated with normal mouse serum (not shown). The immune precipitate of antigens from 2×10^{6} spleen cells was applied to this gel.

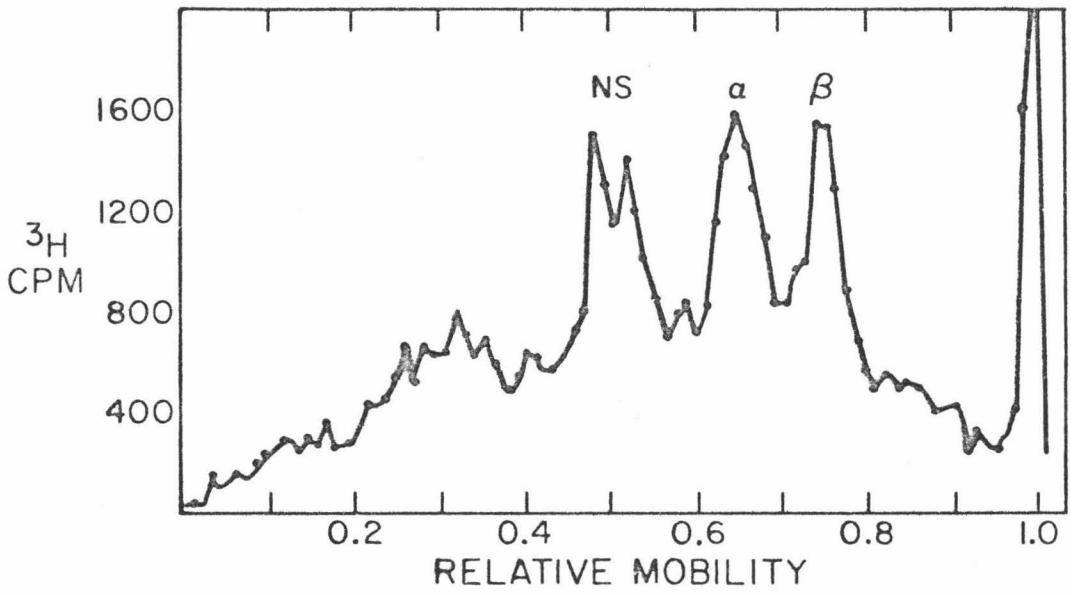

NS
α
β
1600
1200
800
400
^{3}H
CPM
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 3. SDS-polyacrylamide gel patterns of ^{3}H-tyrosine labeled BN antigens.

A) BN antigens detected by R150 rat alloantiserum.

B) BN antigens detected by mouse anti-$\underline{I}^{k}$ alloantiserum (D3b).

C) BN antigens detected by mouse anti-$\underline{I}^{k}$ alloantiserum (D3b) after initial precipitation with R150 rat alloantiserum. The rat antigen preparations were precleared for immunoglobulins and other non-specific peaks by preliminary precipitation with normal mouse serum and SaCI. The peak designated H-1^{n} contains BN major transplantation antigens. Ia antigens are labeled α and β.

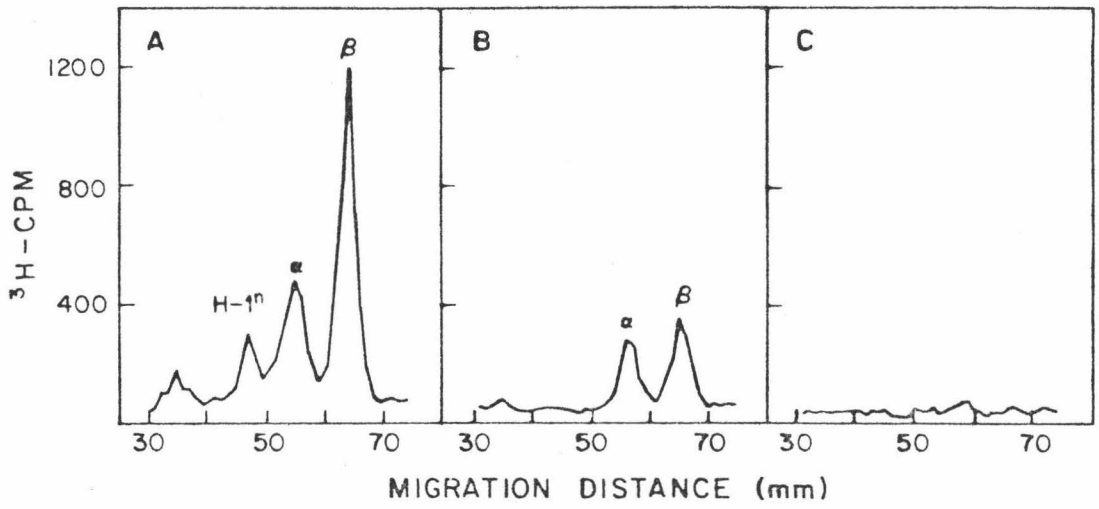

A
B
C
β
α
H-1ⁿ
1200
800
400
^{3}H-CPM
30
50
70
MIGRATION DISTANCE (mm)

Figure 4. Sequential precipitations of ^{3}H-tyrosine labeled BN Ia antigens. BN rat antigens were precipitated with mouse anti-I-EC^{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 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-EC^{k} antiserum (F). This experiment reveals that there are two serologically separable Ia antigens in the BN rat spleen cell preparation.

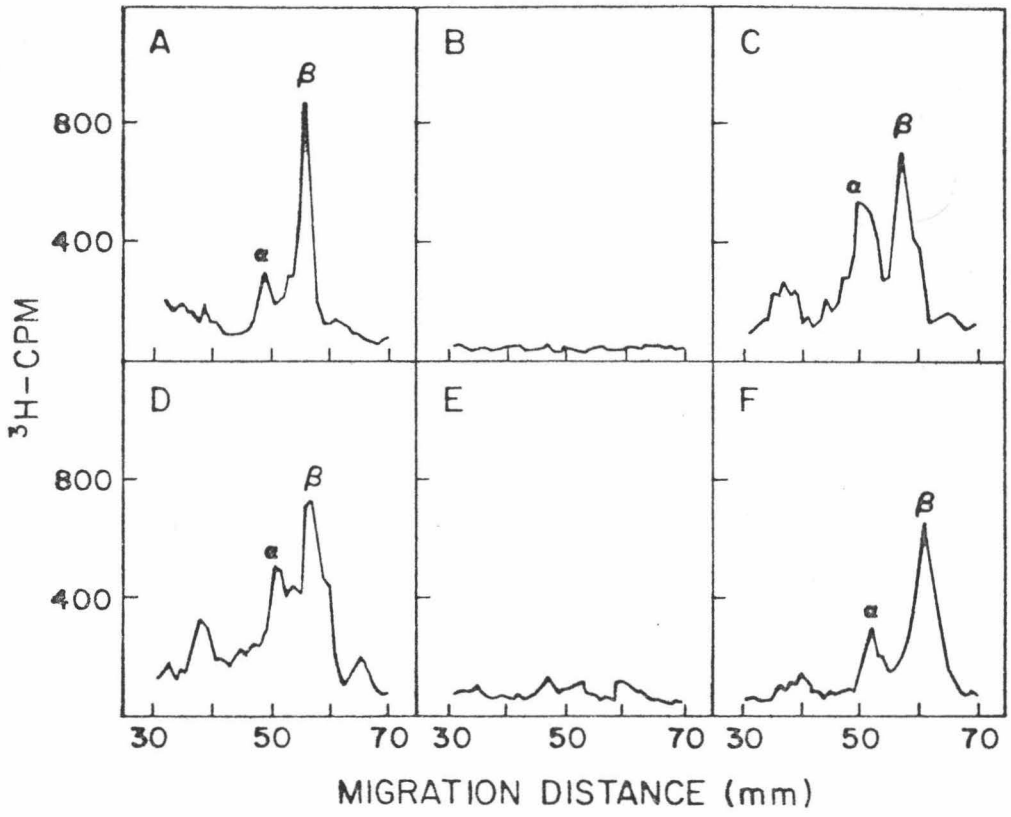

A
B
C
D
E
F
α
β
800
400
^{3}H-CPM
30
50
70
MIGRATION DISTANCE (mm)

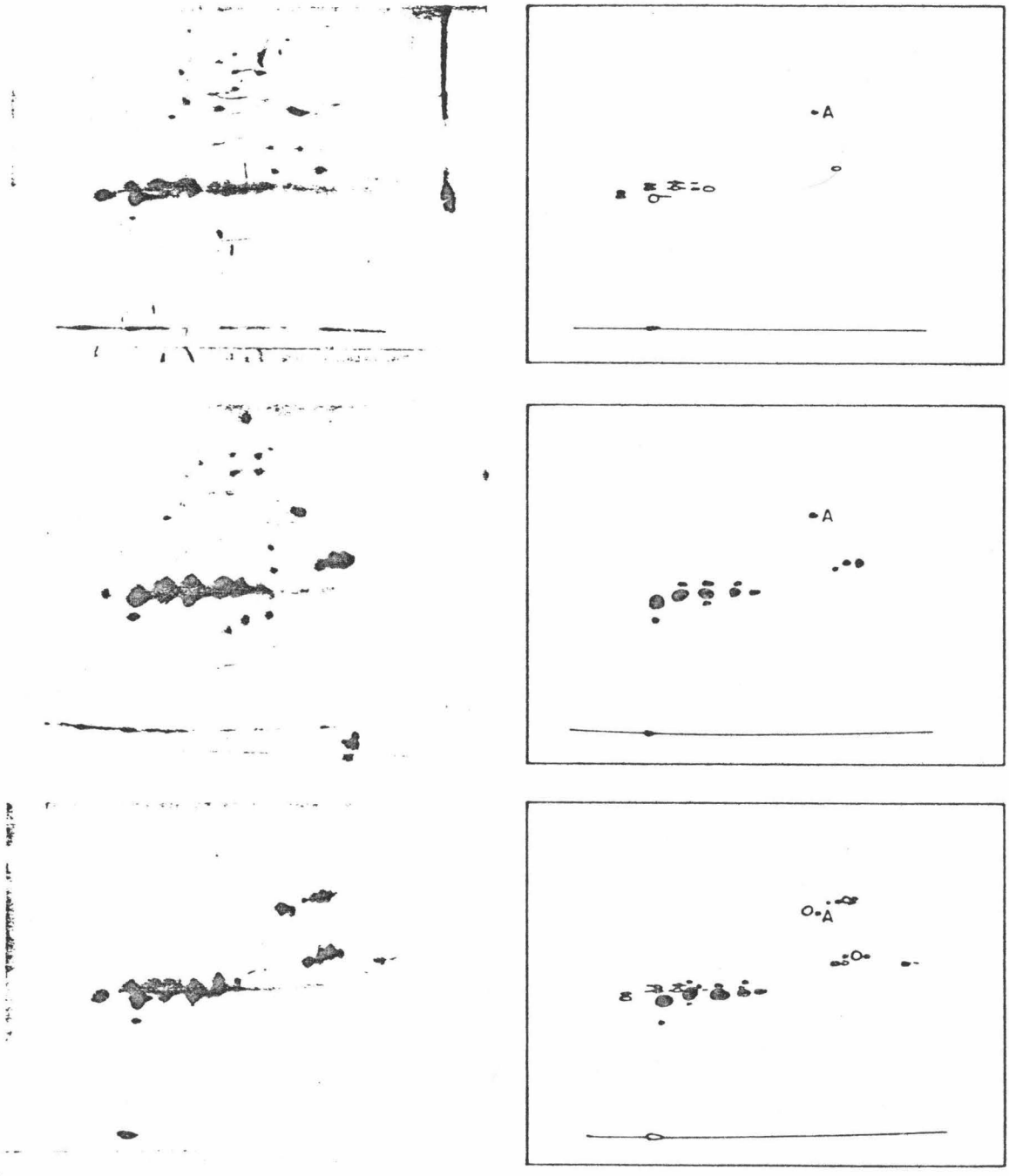
Figure 5. Two-dimensional gel pattern of ^{3}H-tyrosine labeled BN (H-1^{n}) antigens. Non-equilibrium pH gradient electrophoresis (NEPHGE) of immunoprecipitated BN antigens was performed in the first (horizontal) dimension, followed by molecular weight separation on SDS-polyacrylamide gels in the second (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-EC^{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 (45,000 MW), which appear in the vicinity of the actin spot. Beta-2-microglobulin runs at the dye front.


A
A
A

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IX. Immunogenetic Analysis of Alpha Fetoprotein Levels in Developing Mice

Alpha fetoprotein (AFP) is a major serum protein found in mammalian fetuses. It is produced by the embryonic yolk sac and by the developing fetal liver, diminishes greatly in quantity in young animals, and is barely detectable in normal, healthy adults. AFP is also found in large quantities in animals bearing primary hepatic carcinomas, in adults undergoing hepatocyte proliferation after disease or partial hepatectomy, and, in smaller amounts, in maternal serum during pregnancy. (For a concise review of the current research on AFP, see ref. 1; other recent reviews include 2, 3, 4).

The function of AFP is unknown, although several proposals for its abundance in fetal animals have been offered. Two interesting hypotheses state that AFP is either 1) a fetal analogue of albumin which is replaced by albumin during development (1), or 2) an immunosuppressive protein that protects the fetus from immunological attack by the maternal immune system (e.g., see references 5, 6). The latter theory is attractive, but it is controversial (for a review on the immunosuppressive effects of AFP, see 7). There is convincing evidence, in support of the first hypothesis, that AFP resembles albumin rather closely in size (8), charge (9), amino acid composition (8), and in the sequences of several peptides derived from cyanogen bromide digestion (10). When albumin and AFP are chemically denatured, these proteins share immunological determinants (11). Finally, my own work confirms the suggestion of Abelev (12) that there is a reciprocal relationship between the levels of AFP and albumin in the serum of developing fetal mice. After day 15 of gestation, albumin increases and AFP decreases in mouse serum.

It would be interesting to determine the genetic basis for the regular replacement of one protein by another during development. There are two approaches to the study of the genetic control for the expression of AFP. One is to search

for variants that are detectable by one of a variety of methods, including for example, starch gel electrophoresis. Once a structural variation is discovered between two inbred strains, breeding experiments may be conducted to ascertain the chromosomal region containing the gene for AFP. My effort to find either an electrophoretic variant or an antigenic difference among the AFPs of several wild and inbred mouse strains did not reveal any structural variation in this protein at all.

A second way to study the genetic basis for AFP expression is to examine the levels of AFP in mouse strains and in genetically controlled crosses, to determine if there are differences in a possible regulatory gene. During my search for regulatory variants in inbred mice, Olsson et al. (13) reported that such variation does indeed exist. The adult levels of AFP in the BALB/cJ mouse strain are approximately 6 to 10-fold higher than in any of the 26 other mouse strains tested. The authors concluded from their examination of backcross and F2 generation mice that a single recessive gene is responsible for the high level of AFP found in adult BALB/cJ mice.

The experiments in this chapter were designed to determine if the reported regulator of AFP is linked to the structural gene for albumin (14). The assumption I made was that AFP and albumin genes might be found together in one genetic complex, with the regulator for AFP tightly linked to the structural gene for AFP. There is precedent for such linkage in mammals, as the regulator genes for several enzymes, including both beta-galactosidase (15) and beta glucuronidase (16), are closely linked to their respective structural genes in mice. Furthermore, considering the strong homologies between AFP and albumin, it is not unlikely that the structural genes for them are evolutionarily related and might still be

found together within the mouse genome. My results indicate that the control of AFP expression in the mice studied here is not nearly so simple as reported by Olsson (13), and that other factors may be involved in the developmental regulation of this protein.

MATERIALS AND METHODS

Mice: C3H.Alb-1^c mice (14) were a generous gift of Dr. Michael Petras, University of Windsor, Windsor, Ontario, Canada. They were derived from a wild mouse deme bearing the very rare Alb-1^c allele, which was bred onto a C3H background for several generations. They are genetically A B C Alb-$1^{c/c}$, and phenotypically normal (low) for adult AFP expression. BALB/cJ mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and are A b c Alb-$1^{a/a}$ and are high AFP producers as adults. Small numbers of (C3H.Alb-1^c ♀ x BALB/cJ ♂)F1 female mice were crossed to BALB/cJ males to obtain the six backcross litters observed in this study.

AFP and antiserum: A bootstrapping method was used to obtain purified AFP. Homogenates were made from 11-15 day old C3H/HeJ mouse embryos. Crude fractions containing AFP were prepared by precipitation with 60% saturated $(NH_4)_2SO_4$ and separation over G100 chromatographic columns. The broad 70,000 MW peak was collected and used for the first immunization. The rabbit was injected subcutaneously with the mouse fetal antigen preparation in complete Freund's adjuvant, boosted with an aqueous solution of these fetal antigens four weeks later (i.v.), and bled two days later. This anti-mouse embryo serum contained antibodies to mouse AFP and also contained contaminating antibodies to other mouse proteins, including hemoglobin and albumin. The contaminating antibodies

were adsorbed to a matrix of gluteraldehyde-in solubilized adult mouse serum proteins (17) until the antiserum no longer reacted with adult mouse serum on immunoelectrophoresis. The absorbed antiserum from this rabbit was then determined to be specific for AFP by immunoelectrophoresis. The antibodies from this absorbed serum were collected by $(NH_4)_2SO_4$ precipitation and DEAE-sephadex separation, and coupled to Sepharose (18) to make an anti-AFP immunoadsorbant column. A continuing source of AFP for these experiments and for the immunization of other rabbits was prepared using the immunoadsorbant column. The antiserum referred to in this study was prepared against immunoadsorbant-purified C3H/HeJ AFP, according to the immunization protocol outlined above.

Radioimmunoassay: For this study, I developed a radioimmunoassay (RIA) to take advantage of the recent observation that Staphylococcus aureus Cowan fraction I (SaCI) (19) serves as an excellent adsorbant for antigen-antibody complexes. Highly purified mouse AFP (in this case, kindly donated by Dr. E. Ruoslahti, City of Hope Medical Center, Duarte, California) was radiolabeled with ^{125}I by the chloramine-T method (20). ^{125}I was added at a ratio of 1 mCi for 20 µg AFP to be coupled. Unreacted ^{125}I was separated from the labeled protein by gel chromatography over G25 and G100 columns. AFP fractions were diluted 1:1 with 0.1% in bovine serum albumin as a carrier after G25 separation. Graded amounts of purified AFP served as an unlabeled competitor for the binding of ^{125}I-AFP by the rabbit anti-AFP serum. Binding of the competitor (between 2-120 ng) to the appropriate amount of antiserum (usually 100 µl of 1/20,000 dilution) was allowed to take place for 4-6 hours, in the presence of 1/700 heat-inactivated normal rabbit serum. The indicator ^{125}I AFP was added, and the reaction allowed to proceed a further 4-6 hours. In general, the binding of 10 ng

^{125}I-AFP (3.0×10^4 cpm) was assayed. ^{125}I-AFP:anti-AFP complexes were precipitated with 0.5 ml of 3.0% SaCI, and collected by centrifugation. The amount of radioactivity in the pellets and the supernatants was determined on a gamma counter. The percent bound in each sample was corrected for non-specific background (usually 1-2% in the absence of antiserum), and the percent inhibition (%I) was calculated according to the following formula:

$$\%I = \frac{\text{\% bound in the absence of inhibitor - \% bound in presence of inhibitor}}{\text{\% bound in absence of inhibitor}}$$

Serum samples from the individual mice were diluted and values of inhibition for each dilution were compared to the standard curve.

Albumin typing: Albumin phenotypes were determined by starch gel electrophoresis as described (14).

RESULTS AND DISCUSSION

Twenty-seven [(C3H.A1b-1^c x BALB/cJ)F1 x BALB/cJ] mice were assayed for AFP levels at various times over a 10-month period. The results of these assays are given in Figure 1. On the whole, the levels of AFP found in these mice decrease with age, as is expected. There are two groups of mice indicated in Figure 1 -- those with very high levels of AFP and those with low levels. By 30 days after birth, the low AFP producers have reached nearly adult levels of AFP. In contrast, the mice with high concentrations of AFP have levels that are still declining at 220 days. This phenomenon of long AFP persistence resembles that reported by Olsson (13) for high AFP producers. It can also be seen that most of the backcross mice are found to have very high levels of AFP, even at

21 weeks of age. This result is in contrast to the 50% that would be found if only a single recessive were responsible for the high AFP phenotype. In fact, low AFP mice are rare (30%, 8/27) and do not occur randomly throughout the population: four of the eight low AFP mice were found in the same litter. Two mice classified as having low AFP levels were somewhat higher at 60 days than expected from the levels of the other low producers at this age. They are placed in the low AFP category because at 180 days they are indistinguishable from the other low AFP mice. These two mice were from the same litter.

There is no evidence of linkage of the AFP phenotype to the albumin genotype. Of the eight AFP low mice, only 63% (5/8) are heterozygous for Alb-1, indicating the independent inheritance of these traits (Table I). There is no apparent linkage of the low AFP phenotype to c, in agreement with the previous report (13). Four of eight low AFP mice had the C allele and, therefore, the distinction between brown (b) and black (B) could be made. Four of four low mice were b/b, but these numbers are too small to be significantly different from the expected 1:1 ratio.

The high and low AFP phenotypes are stable. Several mice were assayed over an eight-week period or for the whole 10 months, and their AFP levels are shown in Table II. The mice that display a very high level of AFP at 35 days are also high AFP producers at 150 days, 210 days, and at the end of the experiment. Likewise, two female mice (#2 and #6) remained low for AFP production for over five to ten months of the survey, and mice #33 and #31 are low-AFP producers for two samples over a two-month interval. The latter two mice were only two months old at the end of the experiment, so Table II includes only two samples from them.

It has been proposed by Olsson et al. (13), that AFP levels in mice are controlled by a single gene (Raf) with two alleles, the recessive allele (Raf^{h}) being responsible for high serum AFP concentrations, and the dominant allele (Raf^{l}) directing low (or normal) AFP synthesis. One would expect that a backcross generation of mice, produced between an $Raf^{h/l}$ hybrid (with a low AFP phenotype) and an $Raf^{h/h}$ homozygote, would show a 1:1 segregation of this gene and a 1:1 ratio of high AFP:low AFP progeny. In this study, I confirmed that BALB/cJ mice have high AFP levels compared to the presumably normal C3H.$Alb\text{-}1^{c}$ (low AFP) mice, and that at least two hybrid (C3H.$Alb\text{-}1^{c}$ x BALB/cJ)F1 mice displayed the low AFP phenotype. However, the segregation of AFP levels in the backcross generation was 2.4:1 (high:low) and not 1:1.

There are several possibilities that could explain this unexpected result. One is that non-genetic factors are responsible for the high AFP levels found in some of the 19 mice. Diseases of the liver or chemically induced liver damage can account for temporary elevations of serum AFP in experimental animals (22). Although it is possible that some of the progeny were sick, all of them appeared to be healthy over the course of these experiments, and there were no sporadic fluctuations of AFP in any mice tested. Both the high and low phenotypes are stable. The maternal environment could also influence the lifelong AFP concentration in the progeny. I did notice a rather strong litter effect in this study, but this was not consistent between litters from the same mother. Another possibility is that there is an effect of background genes on the level of AFP in mice. The C3H.$Alb\text{-}1^{c}$ mouse strain used in this experiment is not an inbred strain, and a considerable portion of its genome is derived from wild mice. It is not the same strain as the Raf^{l} parent used in the Olsson study. It

would be interesting to see if other genes which affect the levels of AFP could be a factor, but various physiological conditions could result in the synthesis or persistence of high levels of AFP, and genetic analysis of these factors would be a formidable task. The main point is that I found no association of any genes responsible for the final AFP concentration in backcross mice with the gene for albumin (Alb-1), albino (c), or brown (b). This comparison could be extended to more backcross mice to see if the suggestion of linkage between AFP levels and the B/b locus is confirmed.

Table I. Segregation of albumin and levels of AFP in backcross mice

Phenotype	Number of mice
Parental types	
Alb-1$^{a/a}$, high AFP*	7
Alb-1$^{a/c}$, low AFP*	5
Recombinants	
Alb-1$^{a/c}$, high AFP	12
Alb-1$^{a/a}$, low AFP	3

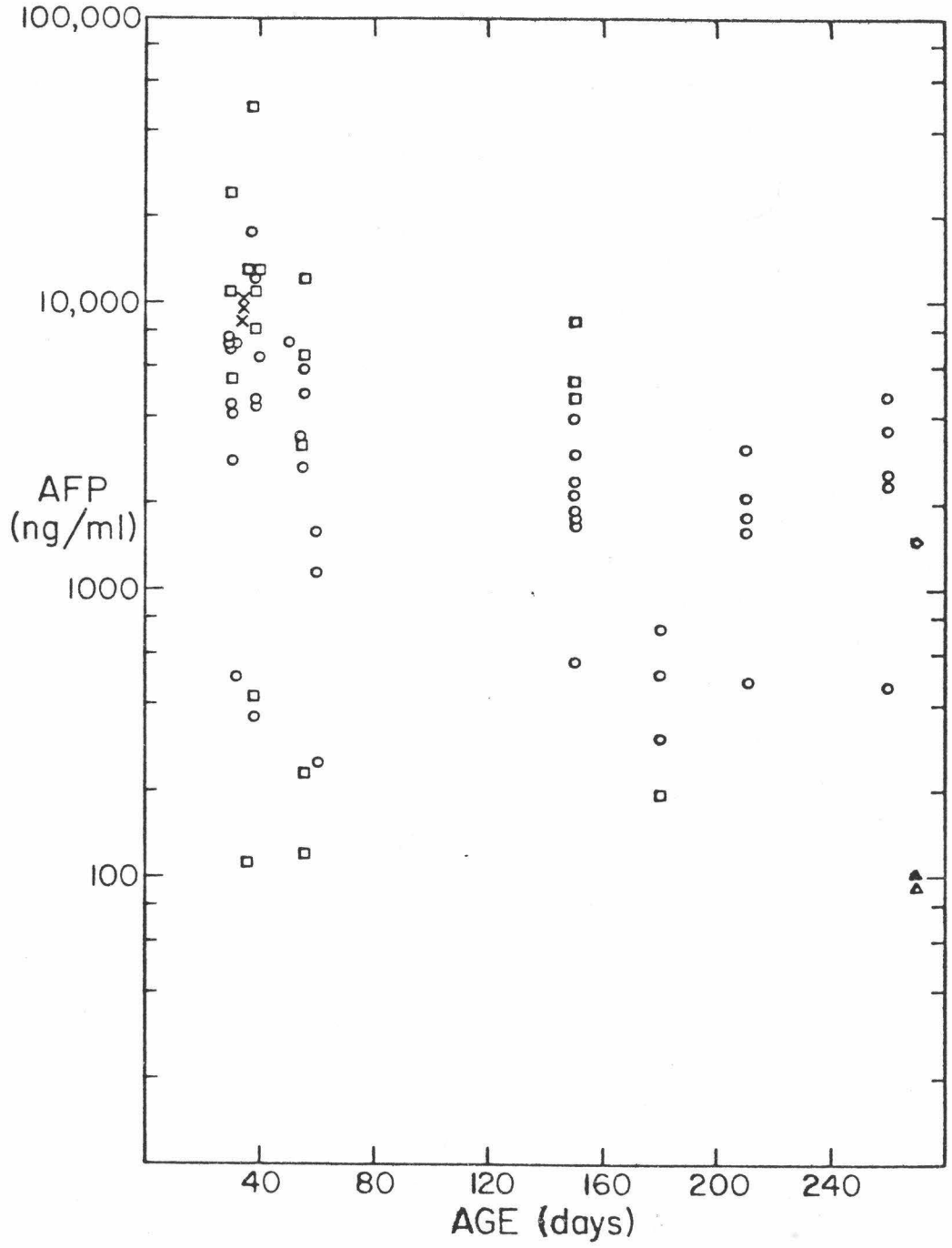
*Backcross mice were typed as high or low AFP producers as described in the text. The average AFP concentration at 50-60 days after birth in high AFP mice was approximately 5500 ng/ml (range: 2600-12,000), and in low AFP mice at the same age was about 600 ng/ml (range: 120-1600).

Table II. AFP phenotype stability in seven individual backcross mice

	ng AFP/ml in serum at age				
Mouse #	30 days	50 days	150 days	210 days	300 days
7	2800	n.d.*	1900	1600	2300
8	6800	n.d.	3000	2100	4800
9	7000	n.d.	1750	1800	3500
10	4500	n.d.	4000	3000	2500
6	500	n.d.	550	480	450
31	100	120	n.d.	n.d.	n.d.
33	440	240	n.d.	n.d.	n.d.

*n.d. = not determined at this age

Figure 1. The concentration of serum AFP in mice over time. Points on this graph represent the sequential sampling of sera from 27 individual backcross mice over a period of 20-260 days. Values are given in nanograms AFP/ml for male backcross mice (□) and female backcross mice (○). In addition, values are given for one adult BALB/cJ male used as one of the backcross parents (◇), one C3H.Alb-1^c female (△), one (C3H.Alb-1^c x BALB/cJ)F1 female that produced one of the backcross litters (▲), and three 35-day-old BALB/cJ mice (×). Backcross mice were assigned to the low AFP category if their serum AFP level was below 2500 ng/ml at days 20-60, and below 1000 ng/ml after day 60.


100,000
10,000
1000
100
AFP
(ng/ml)
40
80
120
160
200
240
AGE (days)

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