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Membrane Molecules Determined by the *H-2* Associated Immune Response Region: Isolation and Some Properties

(histocompatibility/transplantation antigens)

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ABSTRACT **Membrane associated molecules that are probably glycoproteins could be specifically precipitated from NP-40 detergent solubilized extracts of radiolabeled mouse spleen or lymph node cells by antisera produced in congenic strain combinations differing only in the *Ir* gene region which is linked to the *H-2* genes. These *Ir* region products were designated Lna (lymph node antigen) to conform to previous serological work.**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of unreduced specific immune precipitates revealed the presence of a possible dimer form, while reduced samples showed only a single peak equivalent to 30,000 daltons. Thus the Lna molecules are clearly distinct from the *H-2D* and *H-2K* molecules, which are about 45,000 daltons. Anti-Lna antibodies of different specificity can be present in a single serum; there were at least two separate antigen molecules present in one haplotype tested.

The Lna antigens have recently been defined as a system of cell surface antigens which are determined by a gene region closely linked to the *H-2* histocompatibility genes (1). These antigens have been found only on lymphoid cells, and are most easily detected on lymph node cells using cytotoxicity assays. For this reason, the antigen system was called Lna, i.e., lymph node antigen. An antigen system linked to *H-2* but defined by different antisera has also been described recently (2).

The chromosome region bearing the *H-2* genes is complex, but can be subdivided into four regions, each one bearing at least its characteristic gene; *H-2K*, *Ir*, *Ss*, and *H-2D*. In the immunizations which were done to produce the anti-Lna antibodies (1), mouse strains were carefully selected to eliminate *H-2K* and *H-2D* differences, as well as differences extraneous to the *H-2* chromosome segment. Thus the genes for the Lna antigen system were localized to the *Ir* region.

This region is of particular interest because it seems to control several types of immune functions (3, 4). The *MLR* locus, which is also localized to the *Ir* region, appears to control the extent of proliferative response in the mixed lymphocyte reaction (5, 6). The Lna antigen system may or may not be related to one of these functional traits.

MATERIALS AND METHODS

Radiolabeled Antigen. Spleen or lymph node cells were suspended at 2×10^7 cells per ml in labeling medium and incubated at 37° with 5% CO_2. Joklik modified MEM (minimum essential Eagle's medium) with freshly added glutamine but lacking leucine was used to label with [^{3}H]leucine (50 μCi/ml, New England Nuclear Corp., 50–100 Ci/mmol). Complete Joklik modified MEM with freshly added glutamine was used to label with either [^{3}H]galactose, [^{3}H]mannose, or [^{3}H]fucose (50 μCi/ml, New England Nuclear Corp., about 5 Ci/mmol).

After the labeling period the cells were washed and resuspended at 1×10^8/ml in 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris·HCl, pH 7.4, with 0.5% of the nonionic detergent Non-Idet P-40 (NP-40, Shell Chemical Co.). The cell suspension was kept at 4° for 30 min, after which nuclei and debris were removed by centrifugation at $100,000 \times g$ for 60 min. The supernatant material was retained for testing and could be stored at −80°.

Antisera. Immunizations were carried out as described (1). The antisera used in this study were: A.TH anti-A.TL; A.TL anti-A.TH; A.TL anti-A.AL; (C57BL/6 × A/J)F_1 anti-B10.D2; (C57BL/10 × A.CA)F_1 anti-B10.S; (C57BL/10 × AKR.M)F_1 anti-B10.A; (B10.D2 × A/J)F_1 anti-B10.A(5R). The last two antisera were supplied respectively by Dr. G. D. Snell and by the Transplantation Immunology Branch, NIAID, NIH, Bethesda, Md.

The specific anti-Lna antisera (A.TH anti-A.TL and A.TL anti-A.TH) were raised in strains which are identical both in *H-2* type and in the rest of the genome except for any part of the *H-2* chromosome segment inadvertently transferred during crosses to the background strain. The only documented difference between these strains is in the region between the *H-2K* and *H-2D* genes, and in the *Tla* gene which is linked to *H-2D*. Since the Tla antigens are expressed only on the thymocytes, they were not detectable in these studies.

Indirect Precipitation of Radiolabeled Antigen. Radiolabeled antigen preparations were mixed with appropriate antisera and control sera as described previously (7). The soluble antigen–antibody complexes were isolated by precipitation with goat anti-mouse gamma globulin. The precipitates were washed and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The mouse sera were used in excess of labeled antigen when necessary. The goat serum was always used at equivalence.

Pretreatment. Since the material radiolabeled *in vitro* by the spleen or lymph node cells included gamma globulin, the

Abbreviations: MEM, minimum essential Eagle's medium; SDS, sodium dodecyl sulfate; MLR, mixed lymphocyte reaction.

NP-40 extracts were pretreated to remove this endogenous IgG. Carrier normal mouse serum and goat anti-mouse gamma globulin were added to the detergent extracts, and the precipitate which formed carried the [^{3}H]leucine-labeled gamma globulin and was discarded.

Polyacrylamide Gel Electrophoresis. Immune precipitates were dissolved by boiling for 1 min in 2% SDS, or in 2% SDS + 2% 2-mercaptoethanol. The dissociated, or dissociated and reduced precipitates were applied to 0.9 × 10 cm 10% polyacrylamide–SDS gels (8) prepared using a Tris-acetate buffer system and ethylene diacrylate as a cross-linking agent.

Electrophoresis of precipitates was carried out at 15 V/cm for 3 hr. Marker molecules were [^{14}C]leucine-labeled IgG secreted by the mouse myeloma MPC-11 (gift of Dr. M. Scharff). These molecules could be coprecipitated with the tritium-labeled immune complexes by the goat anti-mouse gamma globulin antiserum. [^{14}C]IgG was reduced and alkylated for preparation of H and L chain markers to be used with samples which were not reduced. Alkylation was carried out by adding recrystallized iodoacetamide in twice molar excess of the 2-mercaptoethanol.

RESULTS

Many proteins, including mouse alloantibodies, retain their immunological activity in the presence of the nonionic detergent NP-40, and therefore serological analysis of detergent extracts is feasible. Mouse alloantibody does not ususally give direct precipitation reactions however, so indirect precipitation (i.e., the "piggy back" technique) with anti-mouse gamma globulin has been employed to isolate detergent-solubilized molecules. This method had been shown to be effective for isolation of H-2 antigens and immunoglobulin molecules (7, 9) and was used in the present study for the isolation of Lna antigens.

An NP-40 extract of [^{3}H]leucine-labeled B10.S spleen cells was tested for reactivity with several mouse alloantisera, only one of which had known anti-Lna activity with cells of the type B10.S. Immune complexes were isolated by indirect precipitation, dissolved in SDS and analyzed by polyacrylamide gel electrophoresis (Fig. 1*a* through *d*). Serum A.TL anti-A.TH, shown in panel *c*, produces two major specific peaks of [^{3}H]leucine-labeled material. Their migration rates with respect to reduced and alkylated H and L chains indicate molecular weights of approximately 61,000 and 30,000. The specific nature of these peaks is demonstrated by the fact that they are absent from the precipitates formed with (*a*) normal mouse serum, (*b*) A.TH anti-A.TL (anti-Lna serum of a different specificity), and (*d*) A.TL anti-A.AL (an anti-H-2 serum lacking anti-Lna activity).

When precipitates similar to that shown in Fig. 1*c* were reduced with 2-mercaptoethanol, only a single peak of molecular weight 30,000 was observed. This suggested a dimer/-monomer relationship. Therefore the 61,000 molecular weight species was isolated by fractionation of an SDS-dissolved precipitate of [^{3}H]leucine-labeled B10.S antigen and A.TL anti-A.TH serum on a Bio-Gel 1.5 m column (1.5 × 105 cm) in 0.05 M Tris·HCl, pH 7.4, 0.5% SDS. The isolated 61,000 molecular weight form was subjected to electrophoresis either without further treatment (Fig. 1*e*), or with reduction by 2-mercaptoethanol (Fig. 1*f*). Reduction converted the postulated dimer to the 30,000 molecular-weight species.

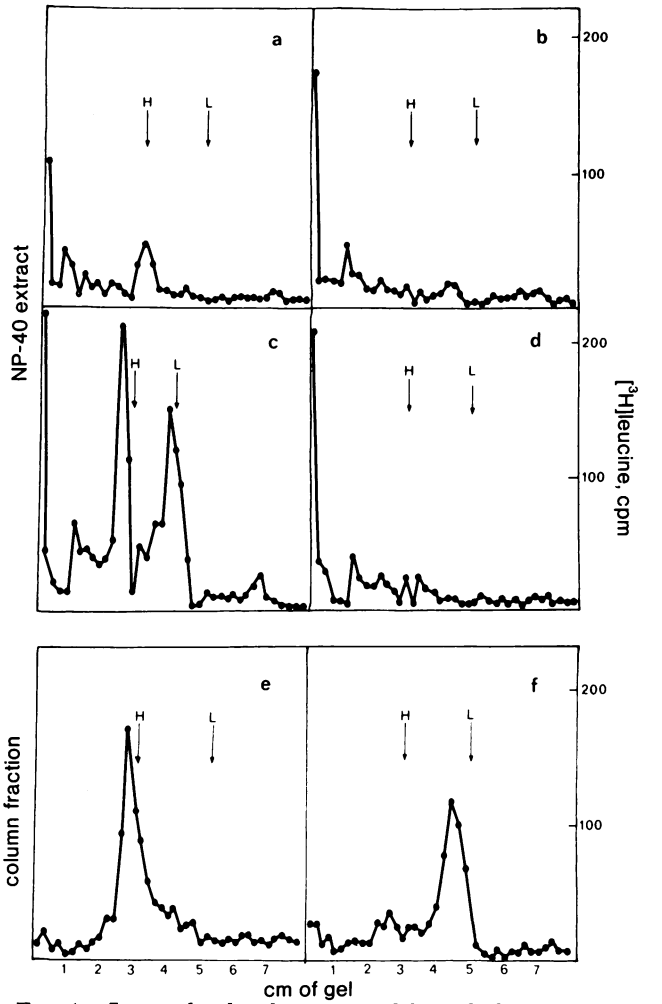

FIG. 1. Lna molecules demonstrated by gel electrophoresis. (*a–d*) Electrophoresis patterns of indirect precipitates of [^{3}H]-leucine-labeled B10.S antigen with four mouse sera: (*a*) normal mouse serum; (*b*) A.TH anti-A.TL; (*c*) A.TL anti-A.TH; (*d*) A.TL anti-A.AL. Precipitates were dissolved in 2% SDS and were not reduced. The positions of marker H and L chains indicate that only the serum A.TL anti-A.TH (*c*) precipitates with molecules showing molecular weights of 61,000 and 30,000 under these electrophoresis conditions. (*e–f*) Results of electrophoresis of unreduced (*e*) and reduced (*f*) aliquots of the 61,000 dalton peak isolated by column chromatography on an SDS–Bio-Gel 1.5 m column.

These experiments showed that the anti-Lna serum which produced a cytotoxic reaction with intact B10.S cells (i.e., A.TL anti-A.TH) was also capable of forming complexes with protein molecules found in radiolabeled B10.S cells.

The specificity of this reaction and its correlation with cytotoxicity tests using the same sera was therefore evaluated. Each antiserum was reacted with detergent-solubilized [^{3}H]-leucine-labeled material from spleen cells of various genotypes. The complexes were isolated by indirect immunoprecipitation and reduced so that only the single peak of about 30,000 daltons would be found on analysis of SDS–polyacrylamide gel electrophoresis. The results of these experiments are given in Table 1. Positive scores are given when a peak was observed in the 30,000 molecular weight region of the gel, as judged by the use of H and L chain markers. Normal mouse serum, and whichever other sera showed no such peaks are given nega-

TABLE 1. *Reaction of anti-Lna and control sera with [^{3}H]leucine-labeled, detergent-solubilized antigen preparations from different strains*

Strain	Antigens: Genotype K	Ir	Ss	D	Antisera* A.TH anti-A.TL	A.TL anti-A.TH	A.TL anti-A.AL	Normal mouse serum
A.TL	s	s	s	d	0	+	0	0
A.TL	s	k	k	d	+	0	0	0
B10.HTT	s	k	k	d	+	+	0	0
B10.S	s	s	s	s	0	+	0	0
B10.BR	k	k	k	k	+	0	0	0
B10.D2	d	d	d	d	+	0	0	0
B10.A	k	k	d	d	+	0	0	0
B10.A(2R)	k	k	d	b	+	0	0	0
B10.A(4R)	k	k	b	b	+	0	0	0
B10.A(5R)	b	b	d	d	+	0	0	0
B10	b	b	b	b	0(+)†	0	0	0(0)†
A.BY	b	b	b	b	0(+)†	0	0	0(0)†

* Antisera A.TH anti-A.TL and A.TL anti-A.TH contain anti-Lna antibodies, but have no anti-H2 reactivity. The A.TL anti-A.AL serum contains anti-H-2K^k antibodies, but no anti-Lna activity.

† Scores in parentheses indicate tests done with 3- to 5-fold greater antigen concentration, judged by quantity of radiolabel.

tive scores. The gel patterns were similar to that shown in Fig. 1*f*.

As shown in Table 1, antiserum A.TH anti-A.TL was positive both with A.TL antigen and with other antigens from strains with dissimilar genotypes. It was negative with antigens from strains which are *Ir^s*, (i.e., have the *Ir* region of the *H-2^s* haplotype). Antiserum A.TL anti-A.TH was positive with A.TH antigen and the other *Ir^s* strain, B10.S. There is one strain, B10.HTT, with which both sera were positive, and none with which both were negative. The control sera, A.TL anti-A.AL and normal mouse serum, were uniformly negative.

This pattern of reactivity is a faithful reproduction of cytotoxicity data obtained with the same sera (1). The only discrepancy which appeared was the initial failure to find reactions between B10 or A.BY and the A.TH anti-A.TL antiserum. When greater amounts of these antigen preparations were used, the activity in question could be detected.

The Lna antigens were originally described as being most easily detected on lymph node cells but it was noted that they were present on spleen cells also. We compared detergent-solubilized extracts of [^{3}H]leucine-labeled lymph node and spleen cell populations to determine whether the antigenic activity detected by indirect precipitation appeared in extracts from both cell sources. Electrophoretic analysis of both spleen and node preparations from A/J mice showed the same 30,000 dalton peak of radiolabel when precipitates made with A.TH anti-A.TL serum were electrophoresed.

Preliminary tissue distribution studies indicate that the Lna antigen appears to be lacking from some tumor cells. Nonradiolabeled NP-40 solubilized extracts from several H-2 bearing tumor cell lines were incapable of blocking the reaction between anti-Lna sera and radioactive spleen antigen preparations. Extracts of Meth-A tumor (*H-2^d* fibrosarcoma), P815 (*H-2^d* mastocytoma), or L5178Y (*H-2^d* lymphoma) were unable to block the reaction of (C57BL/6 × A/Jax)F$_1$ anti-B10-D2 serum against the Lna antigen found in radiolabeled B10.D2 spleen cell extract. On the other hand, the anti-H-2 reaction of this serum was blocked by the unlabeled tumor cell extracts. The assumption made here is that the *Ir* genotype of the tumor cells is the same as the genotype of the source of radiolabeled antigen.

The cytotoxic effect of anti-Lna antisera suggests a cell membrane location for the antigen. Further evidence for a cell membrane association came from experiments which showed that the Lna molecules could be isolated from a crude membrane preparation.

Radiolabeled B10.A(4R) cells were disrupted by hypotonic lysis and a membrane pellet and cell sap fraction were prepared (10). The membrane pellet was resuspended in 0.15 M NaCl, 1.5 mM $MgCl_2$, and 0.01 M Tris·HCl, pH 7.5, and the suspension was made 0.5% in NP-40. After 30 min on ice, the suspension was centrifuged at 100,000 × *g*, and the solubilized material in the supernatant was tested for Lna activity. While the antiserum A.TH anti-A.TL did not react with the cell sap fraction, the precipitate of the material solubilized from membranes showed the specific 30,000 dalton peak on SDS gels. The amount of cytoplasmic fraction tested contained a 5-fold greater quantity of radiolabeled material than the sample of solubilized membranes in which the Lna antigen was observed.

The Lna antigens appear to be relatively heat labile. It was noted that incubation of an NP-40 solubilized [^{3}H]leucine-labeled B10.A spleen cell antigen for about 16 hr at 37° caused a coagulate to form. When this coagulated material was removed the supernatant no longer showed the Lna antigen peak (tested with B10 × A.CA anti-B10.S serum) but still retained active H-2 antigen.

The H-2K and H-2D alloantigens solubilized by NP-40 are glycoproteins bearing fucose, glucosamine, mannose, galactose, and sialic acid (11). To determine whether the Lna antigen was also glycoprotein, we attempted to label it with radioactive monosaccharides which can be used to label H-2 antigens. Electrophoresis of specific precipitates using A.TH anti-A.TL serum and detergent solubilized extracts of B10.D2 (*H-2^d*) cells labeled either with [^{3}H]fucose, [^{3}H]mannose, or [^{3}H]galactose showed incorporation into the Lna antigen peak. When the serum (C57BL/6 × A/J)F$_1$ anti-B10.D2 was used to make precipitates with these B10.D2 antigen preparations, both H-2 and Lna peaks were detected upon SDS gel

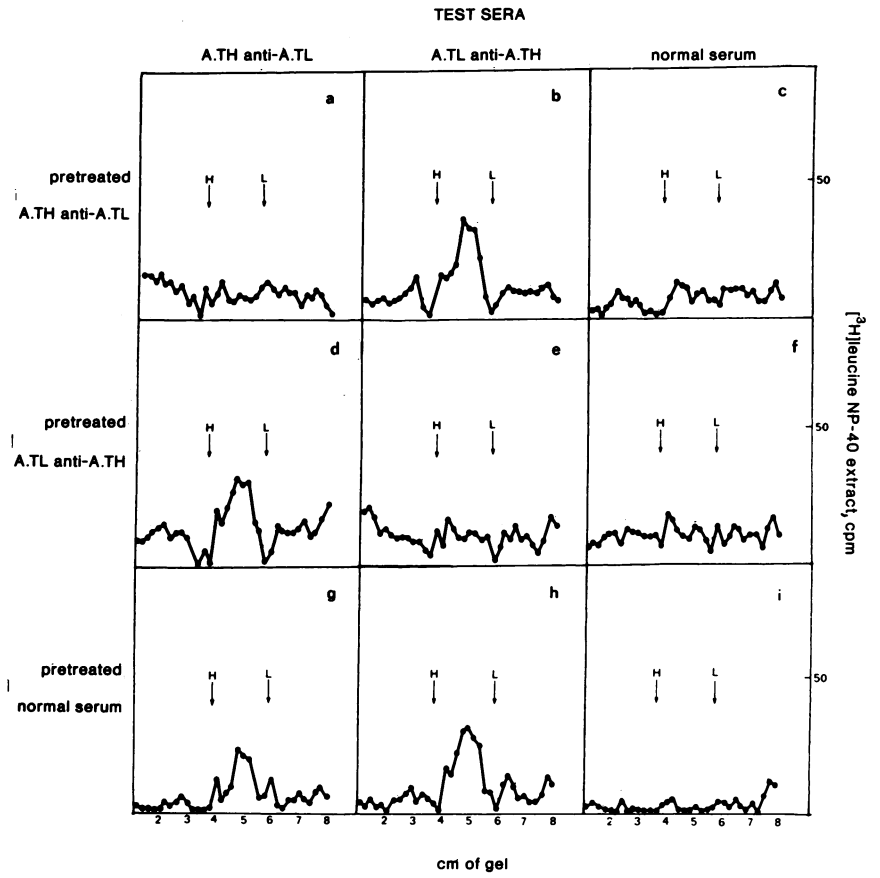

FIG. 2. Demonstration of two different Lna antigen molecules in an extract from strain B10.HTT. Separate aliquots of B10.HTT antigen were reacted with excess A.TH anti-A.TL, excess A.TL anti-A.TH, or normal mouse serum (control). The indirect precipitates made from these three mixtures were removed, and the supernatant materials were each tested for capacity to react with A.TH anti-A.TL, A.TL anti-A.TH, and normal mouse serum. The precipitates from the second round were dissolved in SDS, reduced, and electrophoresed. The supernatant from the A.TH anti-A.TL pretreatment lacked Lna molecules reacting with that serum. This is shown by the absence of a 30,000 dalton peak (between the arrows indicating migration positions of ^{14}C-labeled H and L chains) in the second round test (*a*). However, Lna molecules reacting with A.TL anti-A.TH remained in that supernatant and were complexed in the second round (*b*). Conversely, when Lna molecules reacting with A.TL anti-A.TH were removed by the first round pretreatment (*e*), the other type of Lna molecules were left to react with the A.TH anti-A.TL serum (*d*). The normal mouse serum controls (*g, h, i*) show that Lna reactivity was not disturbed by a control first round of precipitation, and the other controls (*c, f*) show that there was no residual complexed, but unprecipitated material from the first round supernatants precipitated by the second round.

electrophoresis. These preliminary observations strongly suggest that at least some of the Lna antigen molecules in the 30,000 molecular weight peak are glycoproteins.

Thus far, our experiments showed clear differences between H-2 molecules, which are glycoproteins of about 43,000 daltons (12), and the Lna molecules, which are probably glycoproteins of about 30,000 daltons. However, to document further the separate identity of the antigens determined by these linked *H-2* and *Ir* regions, we carried out an experiment showing that H-2K and H-2D molecules could be removed from an NP-40 extract without removal of the Lna antigens. In this experiment, an anti-H-2K^b serum ((B10.D2 × A/J)F$_1$ anti-B10.A (5R)) and an anti-H-2D^d serum ((C57BL/10 × AKR.M)F$_1$ anti-B10.A) were reacted with the B10.A(5R) antigen and after precipitation with goat anti-mouse serum the supernatant was divided and retested with the anti-H-2 sera and with A.TH anti-A.TL. The precipitates were reduced and subjected to electrophoresis. H-2 activity was absent since no peak appeared in the 43–48,000 range with either anti-H-2 serum, but the 30,000 dalton Lna peak was readily demonstrable with the A.TH anti-A.TL serum.

"Anti-H-2" sera also frequently were found to contain antibodies reacting with antigen preparations to give an electrophoretic peak of the same size range as Lna in addition to the H-2 antigen peak. The reactions of several of these anti-H-2 sera indicate that autoantibodies against Lna molecules can be elicited. However, such autoantibodies were not detected in the sera A.TL anti-A.TH or A.TH anti-A.TL using the indirect precipitation assay.

From Table 1, our experience with combined anti-H-2, anti-Lna sera; and the preceding work (1), it is apparent that the anti-Lna antibodies in a single serum frequently can react with more than one *Ir* genotype. This can be an indication that (*a*) single antibody molecules can cross-react with different antigen molecules which share a cross-reactive site, and/or (*b*) that there are different antibody molecules reacting with various antigenic sites on the same molecule or with separate antigen molecules.

In the previous paper (1), absorption studies using the cytotoxicity assay indicated that different anti-Lna antibody populations existed in the same serum. In addition, the existence of at least two separable Lna molecules could be demonstrated in one mouse strain (B10.HTT) by the analysis with the indirect precipitation method. In these studies, removal of Lna molecules reactive with A.TL anti-A.TH antibodies did not remove Lna molecules reactive with A.TH anti-A.TL antibodies, and vice versa (Fig. 2).

DISCUSSION

The antisera used to define the Lna antigens have been used here to isolate a membrane-associated protein molecule which is presumably the *in situ* attachment site for the cytotoxic anti-Lna antibodies. The physiological role of these molecules is presently unknown, but is of particular interest because these entities are determined by the *Ir* region linked to *H-2*, and hence may have some connection to one of the known *Ir* region functions. They may also be related to some as yet unrecognized *Ir* region trait. In any case, their membrane location

assures that they are available for possible interaction with the cell environment.

The data presented here in Table 1 and in the previous paper (1) show that the anti-Lna sera react with the same spectrum of strains whether they are tested by a cytotoxicity assay or by indirect immunoprecipitation of labeled antigen molecules. The titer of antibodies needed to show a positive reaction is greater for the indirect precipitation assay.

The range of reactivity of anti-Lna sera with mouse strains differing in their *Ir* genotype implies antigenic complexity in the system. The Lna peaks on SDS gels are frequently broad and sometimes have shoulders. Absorption studies (1) have shown that there may be several types of antibodies in a given serum, and the experiment presented in Fig. 2 indicates further that a single strain may have more than one antigen molecule. The strain B10.HTT is nominally Ir^k, but its history could have allowed the inclusion of some genetic material from Ir^s, thus explaining its reaction with the serum A.TL anti-A.TH. Data on both Lna cytotoxicity and the mixed leucocyte reaction are consistent with this view (13). If this is true, then there may be more than one genetic locus for Lna antigens. B10.HTT and other strains must be examined further to clarify this issue and determine more exactly the basis for observed heterogeneity.

The presence of anti-Lna antibodies in sera raised for H-2 typing and for experimentation on the biological function of the H-2 system clearly presents problems of interpretation. Sera containing only anti-H-2 antibodies probably cannot be obtained by absorption since most tissues bear H-2 antigens. The tissue distribution of the Lna antigens has not yet been thoroughly examined, but while present on lymph node and spleen cells, the antigen appears to be absent from liver, kidney (1), and from certain tumor cells. Anti-H-2 sera raised by immunization with tumor cells lacking Lna antigens should be specific for H-2. Alternatively, anti-H-2 antibodies may be absorbed from a complex serum by using liver or tumor cells, leaving antibodies specific for Lna. This method should provide an adjunct to the direct production of anti-Lna antibodies in *H-2* identical, *Ir* nonidentical strain combinations which are rare at present. The anti-Lna antibodies in anti-H-2 sera were not noticed in previous work involving indirect immunoprecipitation of H-2 molecules (7, 14, 15) because the antigen sources were usually tumor cells which lacked Lna antigens.

The presence of a possible dimer relationship between Lna molecules is reminiscent of the situation found with detergent solubilized H-2 molecules (12) and perhaps among other membrane components (16). This may be indicative of some requirement for an oligomeric form either to achieve stability in the membrane environment or to establish function there.

The data concerning molecular weight and composition are obviously preliminary, but the Lna molecules are clearly differentiable from H-2 molecules not only on genetic grounds, but on the basis of size and separability by a serological method. One task which remains is to determine whether or not these two groups of antigens should be considered to be fundamentally different. Although they are distinct molecules, the Lna antigens could be just another member of the histocompatibility antigen series which has many members other than H-2K and H-2D.

A correlation between MLR reactivity and the Lna antigens might indicate that they are "histocompatibility antigens" of a special class which are responsible for the immune proliferative response. On the other hand, their antigenic variability may not be important for creating sites, which can be recognized as histo-incompatible, but may be secondary to their possible role as receptor molecules (*Ir* gene products) whose function is to recognize foreign antigens. The possibility that Lna antigens are merely serologically detectable variants of proteins determined in the *Ir* genetic region, but are not related in any way to the immune response or to MLR must not be overlooked. The testing of these alternatives should be greatly facilitated by the analyses which will be made possible by the assay reported here.

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