Partial Characterization of Ia Antigens
From Murine Lymphoid Cells

Ellen S. Vitetta[1], Jan Klein[1] and Jonathan W. Uhr

Irvington House Institute and Department of Microbiology, N.Y.U. School of Medicine, New York, N.Y. 10016, Departments of Human Genetics and Oral Biology, Schools of Dentistry and Medicine, University of Michigan, Ann Arbor, Michigan 48104, and Department of Microbiology, University of Texas, Southwestern Medical School, Dallas, Texas 75235.

Abstract

Congenic anti-Ia antisera were used to bind radiolabelled Ia antigens from cells of various strains of mice of known *H-2* haplotype. The results indicate that Ia antigens are proteins of molecular weight 30,000 to 35,000 daltons. The Ia antigens are distinct from known H-2 antigens as judged by independent immunoprecipitation as well as by molecular weight. Ia antigens are synthesized by, and are present on the surface of lymphoid cells as evidenced by incorporation studies using ^{3}H-leucine and enzymatic radioiodination of cells, respectively. Tissue distribution of cell surface Ia suggests that Ia antigens are on B cells. Ia antigens were detected in the incubation media of ^{3}H-leucine labeled splenocytes suggesting that antigens may be secreted.

Introduction

The *H-2* complex of the mouse can be operationally divided into two peripheral (*K* and *D*) and two central (*I* and *S*) regions.[2] The peripheral regions code for the classical H-2 antigens detectable by serological and transplantation methods; of the two central regions, one (*S*) controls the production of serum proteins (Ss and Slp), and the other (*I*) is involved in the genetic control of immune response, stimulation in mixed lymphocyte culture (MLC) and in graft-versus-host (GVH) reaction (for a review and references, *cf.* Klein 1974). It has recently been demonstrated that cross-immunization of congenic lines differing in the central *H-2* regions leads to the production of antibodies against antigens that differ from the classical H-2 antigens in that they have a much more restricted tissue distribution (Hauptfeld *et al.* 1973; David *et*

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al. 1973). The antigens, summarily designated Ia (for *I* region-*a*ssociated antigens, *cf.* Shreffler *et al.* 1974) have so far been detected only on lymphocytes and primarily on bone marrow-derived (B) lymphocytes (Hämmerling *et al.* 1974; Hauptfeld *et al.* 1974), although there is some evidence to suggest that they may also be present on epithelial cells (Klein *et al.* 1974). In this communication we present evidence that H-2 and Ia antigens also differ in their physico-chemical properties.

Materials and Methods

Mice

1. B10.T (6R)(K^q *Ir-1A*^{q} *Ir-B*^{q} S^q D^d)(=6R)
2. B10.AQR(K^q *Ir-1A*^{k} *Ir-1B*^{k} S^d D^d)(=AQR)
3. B10.S(7R)(K^s *Ir-1A*^{s} *Ir-1B*^{s} S^s D^d)(=7R)
4. A.TL(K^s *Ir-1A*^{k} *Ir-1B*^{k} S^k D^d)

Sera All immunoprecipitations were done by a sandwich procedure (Vitetta *et al.* 1973). Binding sera included:

1. Rabbit anti-mouse Ig(=RAMIG).
2. 6R anti-AQR(= anti-Ia.1).
3. AQR anti-6R(= anti-Ia.2).
4. (B10 × A.TL) F_1 anti-HTT(= anti-Ia.3, 4).
5. Rabbit anti-mouse Ss (DBA/2)(= anti-Ss).
6. C3H anti-C3H.Q(= anti-Slp).
7. (A × B10) F_1 anti-B10.S(= anti-H-2K.19).
8. (A.BY × B10.AKM) F_1 anti-B10.A(= anti-H-2D.4).
9. Normal mouse serum contained 1:1 mixtures of sera from adult AQR and 6R mice or 7R and B10 mice (= NMS).

All immune complexes were precipitated with an excess of goat anti-rabbit Ig (GAR) or goat anti-mouse Ig (GAM).

Radiolabelling of cells. Spleen cell suspensions were prepared in cold phosphate buffered saline, pH 7.3 (PBS) (Vitetta *et al.* 1971), washed once in PBS and viabilities were determined (Vitetta *et al.* 1971).

a. *Enzymatic radioiodination.* 1-3 × 10^8 splenocytes were radioiodinated with 2-6 mCi of Na^{125}I (New England Nuclear) (Vitetta *et al.* 1971). Reactions were terminated by the addition of 20-30 volumes of cold PBS and cells were washed once in PBS.

b. ^3H-*leucine.* 1-5 × 10^8 cells were washed once in Eagle's minimal essential medium (Grand Island) (MEM) lacking leucine and containing 10% fetal calf serum and 2% antibiotics. Washed cells were suspended at a concentration of 10^7/ml in the same medium containing 20 μCi L-leucine 4, 5-^{3}H/ml (30 Ci/mM) (New England Nuclear) and incubated for 3-5 hours in a moist CO_2 environment. At the end of the labelling period, the cells were separated from the medium by centrifugation and

washed twice in PBS. Nonidet P-40 (NP-40) (Shell Oil Co.) was added to the incuba-
tion medium to a final concentration of 0.5% and the medium was dialyzed for 16 hrs.
at 4°C.

Preparation of cell lysates. Cells previously labelled with ^{125}I or ^3H-leucine
were lysed in 2-4 ml of PBS containing 0.5% NP-40 and 50,000 units of Trasylol
(Bayer) at 4°C for 15 minutes. Nuclei and insoluble debris were removed by centrifu-
gation at 1000g for 15 minutes and the lysates were dialyzed for 16 hours at 4°C
against PBS containing 1×10^6 units/liter Trasylol.

Immunoprecipitation. Following dialysis, lysates were centrifuged at 10,000g
for 30 minutes and total acid precipitable radioactivity was determined in 5% trichlor-
acetic acid (TCA) (Vitetta *et al.* 1971). Labelled Ig was then precipitated from the
lysates by the addition of appropriate volumes of RAMIG +GAR. Precipitates were
washed three times in PBS and the first wash was added to the supernatant.

Ig-depleted lysates from iodinated cells were further treated with 50 μl of
NMS+GAM. This step was necessary to remove labelled surface antigens reacting with
autoantibodies in the mouse sera; it was not necessary when lysates from ^3H-leucine-
labelled cells were used. The final supernatant of the lysates was centrifuged at
10,000g for 30 minutes and divided into three aliquots. These were treated with 100
μl of either NMS, anti-Ia.1 or anti Ia.2 and an excess of GAM. Precipitates were spun,
washed three times in PBS and dissolved in 1% sodium dodecyl sulfate (SDS) contain-
ing 8M urea and 0.2 M mercaptoethanol (ME) at pH 8.4 at 56°C for 60 minutes.

Samples were counted (Vitetta *et al.* 1973) and electrophoresed on 5% SDS
acrylamide gels (Shapiro *et al.* 1967). Gel fractions were counted to 5% error on a
Beckman LS250 liquid scintillation counter. Appropriate markers were electro-
phoresed on companion gels.

Results

Immunoprecipitation with RAMIG. Lysates of cells which had been radio-
iodinated or labelled with ^3H-leucine contained respectively 5 to 30 and 1 to 3×10^6
acid precipitable counts per 10^8 cells. In lysates of iodinated cells, 7 to 9% of the
radioactivity was precipitated with RAMIG and in lysates of ^3H-leucine labelled cells
1 to 4% of the radioactivity was precipitated with RAMIG+ GAR. Reprecipitation of
supernatants was routinely done to assure that all radiolabelled Ig had been removed
by the first step. Further precipitation of lysates from radioiodinated cells with
NMS+GAM removed an additional 4 to 7% of the radioactivity.

^3H-leucine labelled cells. Fig. 1 shows a representative experiment using lysates
of AQR cells labelled with ^3H-leucine. As can be seen, there is a major peak of radio-
activity obtained with specific anti-Ia serum (anti-Ia.1) that is not present using anti-
serum against the congenic partner (anti-Ia.2) or normal serum. The molecular weight
of the antigens in the major peak is approximately 30,000 to 35,000 daltons. Several
minor peaks were obtained with both specific and control antisera containing antigens
with molecular weights of 45,000 to 65,000 daltons and 15,000 to 25,000 daltons.
The minor peaks may represent antigens reacting to autoantibodies contained in both
antisera and occasionally observed in normal mouse sera. The amount of acid pre-
cipitable radioactivity that is specifically brought down by anti-Ia serum (subtracting

the value of radioprecipitation by normal mouse sera) was 0.2 to 0.5% in three experiments.

The incubation media of ^{3}H-leucine labelled splenocytes were also examined for radioactive molecules which were reactive with anti-Ia sera. Fig. 2 shows an experiment in which 7R splenocytes were labelled for five hours. As can be seen, when anti-
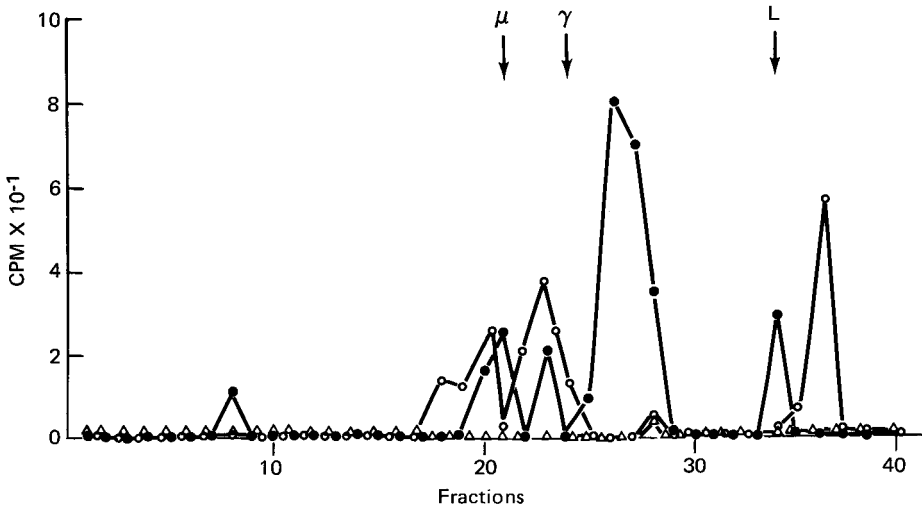

Fig. 1. ^{3}H-leucine-labelled Ia antigen in lysates of AQR (Ia.1) splenocytes. Immunoprecipitates were reduced and alkylated before electrophoresis in SDS-5% acrylamide gels. (●————● = anti-Ia.1; ○————○ = anti-Ia.2; and △————△ = NMS).
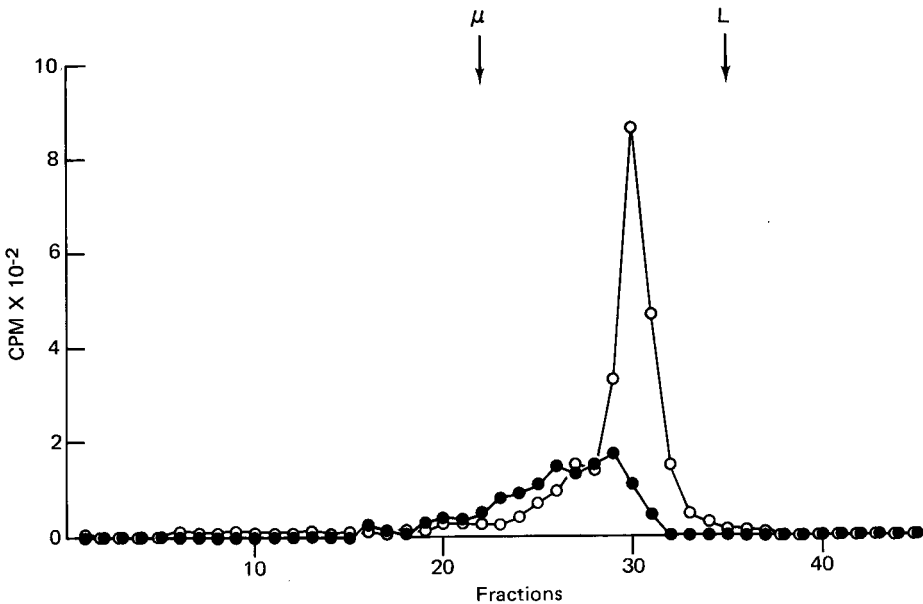

Fig. 2. ^{3}H-leucine-labelled Ia antigen in the incubation media of 7R (Ia.3,4) splenocytes, See Fig. 1. (○————○ = anti-I.a.3,4) and ●————● = NMS.

Ia.3,4 was used, the incubation medium showed a similar major peak to that observed in Fig. 1; normal serum appeared to bind a small portion of these antigens.

   ^{125}I-labelled cells.   It was important to determine if the antigens were present on the surface of splenocytes. For this purpose, cells were enzymatically iodinated and the lysates were immunoprecipitated with appropriate antisera. Fig. 3 shows the results of a "checkerboard" experiment. As can be seen, a series of peaks of molecular weights 45,000 to 65,000 daltons and 15,000 to 25,000 daltons were obtained with
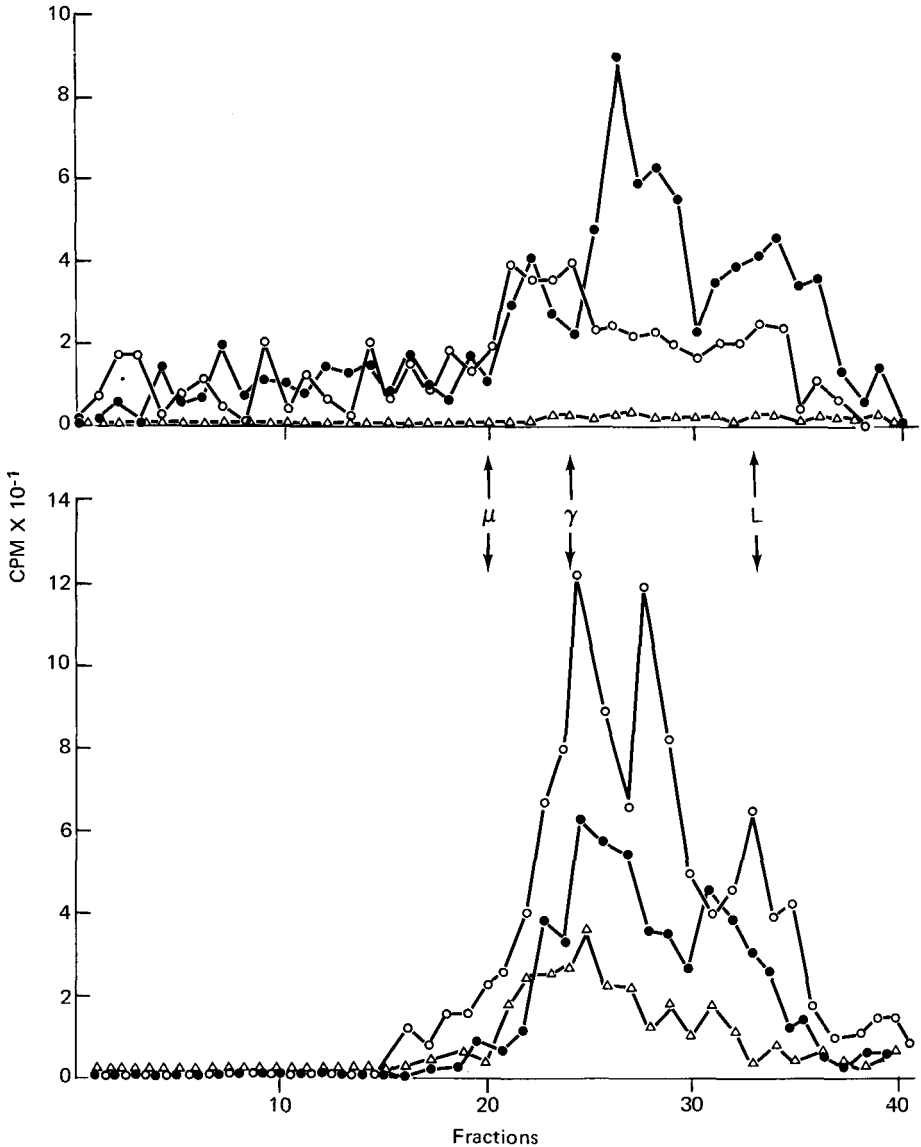

Fig. 3. ^{125}I-labelled Ia antigen in lysates of radioiodinated AQR (Ia.1) (upper panel) or 6R (Ia.2) (lower panel) splenocytes. (●——————● = anti-Ia.1; ○——————○ = anti-Ia.2; and △——————△ = NMS).

both specific and control sera. However, the peak corresponding to approximately 30,000 to 35,000 daltons was again obtained only with corresponding anti-Ia serum. This experiment indicates that the Ia antigen as well as the antigens reacting with the presumed autoantibodies are present on the cell surface.

Tissue distribution of cell surface Ia antigens. As seen in Table 1, the percentage of total cell surface protein which is Ia corresponds to the proportion of B cells in the particular tissue used. This finding suggests that Ia antigens are on the surface of B cells; it does not exclude their presence on thymus-derived (T) cells. The experiments also emphasize the small amount of the acid precipitable radioactivity on the surface of spleen cells that is Ia (0.33%). This value is 11 to 33% of that observed for cell surface H-2 antigens and 2 to 4% of that obtained for surface Ig.

Distinctiveness of Ia antigens and other products of the H-2 complex. To establish that the antigen precipitated by specific anti-Ia sera was not H-2D, H-2K, Ss or Slp, lysates of ^{125}I-labelled 6R cells which had been previously "cleared" with

Table 1. Tissue Distribution of Ia Antigens on Radioiodinated Cells of AQR Mice.

Tissue	Acid precipitable radioactivity/10^8 cells (CPM $\times 10^6$)	% of total acid precipitable radioactivity in Ia antigens *
Bone Marrow	9.06	1.30
Spleen	4.99	0.33
Lymph nodes	9.11	0.23
Thymus	11.12	0.03

$$* \ \frac{100 \ (\text{CPM in anti-Ia precipitate} - \text{CPM in NMS precipitate})}{\text{CPM in acid precipitate}}$$

Table 2. Effect of Pretreatment With Antiserum to Other Products of the *H-2* Complex on Removal of Ia From Lysates of ^{125}I Labelled 6R Splenocytes

Aliquot**	Pretreatment	Ia antigens*** (CPM)
1	Anti-Ss	4649
2	Anti-Slp	4729
3	Anti-(H-2D.4+ H-2K.19)	4906
4	NMS	4235

**Each sample contained 4.52 $\times 10^6$ and precipitable CPM.
***See formula under Table 1.

RAMIG +GAR and NMS +GAM were divided into aliquots and treated with antiserum to other products of the *H-2* complex or with NMS. Complexes were precipitated by the addition of GAM or GAR and the supernatants were examined for the presence of Ia antigens by the subsequent immunoprecipitation with either specific anti-Ia serum or NMS. As shown in Table 2, none of the antisera was more effective than NMS in removing the Ia antigens.

Discussion

The experiments reported here indicate the following conclusions: First, Ia antigens are distinct from classical H-2 antigens, because (a) Ia antigens have a different molecular weight (30,000 to 35,000 daltons as compared to 45,000 daltons or more for H-2 antigens (Nathenson 1970); (b) prior immunoprecipitation of H-2 antigens does not affect subsequent detection of Ia antigens; and (c) Ia antigens, unlike H-2 antigens (Wernet *et al.* 1973) may be secreted. Second, the Ia antigens like H-2 antigens, are located on the cell surface as shown by enzymatic radiodination of intact cells. This conclusion is in agreement with the observation that the antigens participate in membrane-mediated cytotoxicity (Hauptfeld *et al.* 1973; David *et al.* 1973). Third, Ia antigens are present on B cells, because there is a correlation between quantity of radioactive Ia antigen with the proportion of B cells among the various lymphoid tissues studied. This conclusion is also in agreement with previous serological results which demonstrated predominant expression of Ia antigens on bone-marrow derived lymphocytes (B) (Hämmerling *et al.* 1974; Hauptfeld *et al.* 1974).

We have thus established that the *I* region of the *H-2* complex codes for a new class of antigens which are clearly distinct from classical H-2 antigens. The relationship of these new antigens to the *Ir* gene product and to the products of the *Lad* loci which are responsible for lymphocyte activation in MLC and GVH reactions remains to be resolved. Ia antigen is different from the antigen recognized by human sera that inhibit MLC reactions (Wernet and Kunkel 1973) because the latter antigen has a different molecular weight and is on T cells.

The isolation of Ia antigens presented differences from similar studies of H-2 antigens (Schwartz and Nathenson 1972; Vitetta *et al.* 1972; Wernet *et al.* 1973): First, a very small percentage of radioactivity was obtained in the specific immunoprecitate. Second, immune complexes formed with control sera (including NMS) appeared to bind a proportion of Ia molecules. Third, Ia antigens were present in the incubation medium. These findings could all be related. Thus, if Ia is shown to be actively secreted, much of the antigen may be extracellular in a long term labelling experiment. In addition, if Ia has affinity for immune complexes, the majority of Ia could be lost in the prior immunoprecipitations designed to remove mouse Ig. Analyses by acrylamide gel electrophoresis of the reduced immunoprecitates obtained with RAMIG disclosed an additional peak of radioactivity that migrated like Ia. Double label experiments would be necessary to test further whether this peak is Ia, and if so, whether the immunoprecipitation is nonspecific. The possibility of non-specific immunoprecipitation of Ia raises questions concerning the specificity of the immunoprecipitates presented in this report. It could be argued that increased amounts of particular classes of Ig or the presence of immune complexes in the anti-Ia serum

could have increased its capacity to bind the molecule which we have identified as Ia. Further studies are required to answer these questions.

The presence of Ia in the incubation media raises additional important questions: Is it possible that the antigens secreted in the medium are products of the *S* rather than the *I* region? We consider such a possibility unlikely, because antiserum (B10 × A.TL) F_1 anti-HTT was produced in an *H-2* haplotype combination in which the donor and the recipient did not differ in the *S* region. If Ia antigens are secreted, can they act as signals between different subpopulations of lymphocytes, *e.g.* mediating helper function, lymphokine-like activities, etc? In addition to the possible biological significance of secreted Ia antigens their presence in the media may simplify their biochemical isolation and characterization.

Acknowledgments

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