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Ia ANTIGENS ON MURINE LYMPHOID CELLS: DISTRIBUTION, SURFACE MOVEMENT AND PARTIAL CHARACTERIZATION*

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SUMMARY

Antisera directed against the I (immune response gene) segment of the murine major histocompatibility complex were prepared, and the distribution of lymphoid cells binding the antisera studied by radioautography. A clear bimodal distribution of grain counts was observed in lymph node and spleen. Bone marrow lymphocytes showed heavy but heterogeneous labelling, while thymocytes were lightly but definitely labelled. Approximately 30% of lymph node cells and 60% of spleen cells were heavily labelled. The heavily labelled cells were shown to be B cells. Ia antigens are present on plaque forming cells, but probably in smaller amounts than on resting B cells. One plasma cell tumour was studied, and was found to possess little or no Ia antigen.

There was no demonstrable labelling of thoracic duct lymphocytes from preparations of T cells activated to histocompatibility antigens. However, several T cell lymphomas probably possess Ia antigens, since they were rejected when transplanted into mice differing at the I region. Overall, the data indicate that Ia antigens are expressed on at least some T cells.

The Ia antigens were shown to move independently of surface immunoglobulin on B cells, demonstrating the non-identity of these molecules. Pretreatment of B cells with anti-immunoglobulin did not inhibit binding of anti-Ia sera. The Ia antigens could be capped by a sandwich technique in 75% of labelled cells.

The Ia antigens were specifically precipitated from lysates of surface radioiodinated spleen cells, and the products analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. A single specific peak of radioactivity corresponding to a molecular weight of approximately 30,000 was

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found. The significance of these results in relation to immune response genes, receptors for antigen on T cells, and receptors for the Fc portion of IgG on B cells, is discussed.

INTRODUCTION

In many species, the immune response to certain antigens is under the control of specific 'immune response' (*Ir*) genes which are closely linked to the major histocompatibility complex (Benacerraf & McDevitt, 1972; Shreffler & David, 1974). These genes apparently show dominant inheritance and control specific immune responsiveness at the T cell level, although their precise mode of action has not been determined. It has been proposed (Benacerraf & McDevitt, 1972) that these genes represent structural genes for a non-immunoglobulin receptor for antigen on T cells. However, evidence for such an hypothesis is indirect, and other models for *Ir* gene function have been proposed (Marchalonis *et al.*, 1974; Katz & Benacerraf, 1974).

Recently, congenic strains of mice have become available which differ at the H-2I chromosomal segment, but are identical at the H-2K and H-2D loci (Shreffler & David, 1974). When mice of one strain are immunized against lymphoid cells of the partner, antibodies cytotoxic to lymphocytes are produced in high titre (David *et al.*, 1973; Hauptfeld *et al.*, 1973). The antigens detected by these antisera are independent of the H-2K and H-2D antigens, and have been termed Ia (I region associated) antigens (Shreffler *et al.*, 1974).

In view of the apparent functional expression of H-2 linked *Ir* genes in T cells, it was expected that Ia antigens would also be expressed in T cells. However, evidence has been presented for exclusive B cell localization of Ia antigens (Hammerling *et al.*, 1974; Sachs & Cone, 1973), exclusive T cell localization (Gotze *et al.*, 1973) and expression on both cell types (Frelinger *et al.*, 1974b).

As a preliminary approach to the question of the relationship between Ia antigens and products of the H-2 linked immune response genes, we have set out to determine whether anti-Ia antisera bind predominantly to T cells, B cells or both, using radioautography and a variety of lymphoid cell populations. In populations where a mixture of T and B cells were present, a bimodal distribution of grain counts was observed, and the heavily labelled cells were shown to be B cells. Weaker, but definitely significant binding was also observed in thymocytes. The antigens were shown to be non-immunoglobulin in nature, as they did not co-cap with immunoglobulin on B cells. They have an estimated subunit molecular weight of approximately 30,000.

MATERIALS AND METHODS

Mice

A.TH and A.TL mice were progeny of breeding pairs obtained from the Department of Human Genetics, University of Michigan, and were used at an age of 6–8 weeks. Their H-2 genotypes are:

Strain	K	I	S	D
A.TH	s	s	s	d
A.TL	s	k	k	d

CBA/H mice (H-2^k) and CBA × C57Bl F_1 mice (H-2^{kb}) were obtained from colonies maintained at the Walter and Eliza Hall Institute by Dr M. C. Holmes.

Congenitally athymic ('nude') mice in their fourth generation of backcrossing to CBA mice, and possessing the H-2^k histocompatibility complex, were provided by Dr M. C. Holmes.

Antisera

A.TH anti A.TL serum was prepared as described by David *et al.* (1973). Briefly, A.TH mice were injected four times intraperitoneally with a mixture of 2×10^6 A.TL thymocytes and 6×10^5 A.TL lymph node cells at weekly intervals, followed by monthly booster injections until a high cytotoxic titre was obtained. The serum was partially purified by starch block electrophoresis (Kunkel, 1954). The gamma globulin fraction was concentrated to 1 mg/ml by pressure dialysis.

A strong polyvalent rabbit anti-mouse IgM serum was prepared as previously described (Nossal *et al.*, 1972).

Anti Thy 1.2 (previously anti θ) serum was prepared by the method of Reif & Allen (1963), and was tested for cytotoxicity against CBA thymocytes and CBA plaque forming cells. Any batches showing B cell reactivity were extensively absorbed with 'nude' mouse spleen cells. In order to eliminate Thy 1.2 positive cells, $10–30 \times 10^6$ viable cells were suspended in 1 ml antiserum and incubated at 37°C for 30 min. They were then washed, and resuspended in 1·5 ml agarose absorbed guinea-pig complement (final dilution 1:6). After incubation at 37°C for 30 min, the cells were washed twice.

Cell suspensions

These were prepared essentially as described by Nossal *et al.* (1972). Care was taken to avoid removing parathymic lymph node with thymus tissue. Lymph node specimens included nodes from the inguinal, axillary and mesenteric groups. In each case, cells from three to four mice were pooled. Dead cells were removed by the method of von Boehmer & Shortman (1973). This method was shown by the authors to have a very high recovery of live cells, and no selective loss of T cells, B cells or macrophages. Typical viabilities as assessed by eosin exclusion were 93–98 %.

Radio-iodinations

Rabbit anti mouse IgM was radio-iodinated with ^{125}I by the chloramine-T method (Greenwood *et al.*, 1963) to a specific activity of 12 μCi/μg, and used as a concentration of 0·2 μg/ml.

In order to minimize denaturation of mouse antibodies, anti-Ia antisera were trace labelled with ^{125}I by the lactoperoxidase method (Marchalonis, 1969). Specific activity was approximately 3 μCi/μg. Unbound iodide was removed by gel filtration. Iodinated anti-Ia antisera were absorbed for autoantibodies by mixing with an equal volume of thymus and spleen cells from A.TH mice at 4°C for 1 hr. Cells were then removed by centrifugation at 400 ***g*** for 5 min. The antisera were then centrifuged at 10,000 ***g*** for 1 hr to remove particulate matter. Radio-iodinated antisera were stored at 4°C and used within 4 days of preparation.

Radioautography

This was performed as previously described (Nossal *et al.*, 1972). 5×10^6 cells were held at 4°C for 30 min with radiolabelled antiserum and then washed through fetal calf serum

gradients and smeared onto gelatin coated glass slides. The slides were then dipped in NTB-2 photographic emulsion, exposed, developed, and stained with Giemsa. Routinely, anti-Ia serum was used at a concentration of 25 μg/ml, and slides were exposed for 3 days. Between 300 and 1000 cells were counted per slide. In some cases, grain counts were performed. In others, cells with more than seven grains were scored as labelled. Only morphologically intact cells resembling lymphocytes were scored. Damaged cells and areas of high background were not scored.

Ia antigens on plaque forming cells

CBA (H-2^k) mice were immunized with 4-hydroxy-3-iodo-5-nitrophenyl acetic acid conjugated to polymerized flagellin (NIP-POL) as previously described (Schlegel, 1974). After 3 or 7 days, mice were killed, their spleens removed and assayed for haemolytic plaque forming cells. Cell suspensions were diluted to give 50–200 plaques per chamber. Enough cells to fill six chambers (typically 10^7) were held with 200 μl undiluted A.TH and A.TL antiserum for 30 min at 4°C, washed, and incubated for a further 30 min at 37°C with selected nontoxic rabbit complement diluted 1:12. They were then tested for anti-NIP plaque forming cells, using sheep red cells coated with NIP conjugated $(Fab)_2$ fragments of rabbit anti-sheep IgG. Controls included complement only, A.TL anti A.TH serum (negative controls) and Balb/c anti CBA serum (positive control).

*C*1.18 *tumour*

This plasma cell tumour (also known as X5563) arose in a C3H (H-2^k) mouse (Potter *et al.*, 1957), and is maintained in tissue culture at the Walter and Eliza Hall Institute by Dr A. W. Harris.

Cell electrophoresis

This was performed according to von Boehmer (1974). This method separates cells on the basis of charge, T cells being more negatively charged than B cells, and, therefore, migrating further towards the anode. Spleen cells from A.TL mice were processed, and each fraction divided into two equal parts. One was exposed to ^{125}I A.TH anti A.TL serum, and the other to ^{125}I anti IgM serum. The cells were then processed for radioautography.

Activated T cells

CBA × C57Bl F_1 mice were irradiated with 850 rad, and then injected intravenously with 100×10^6 CBA thymocytes. Four days later, the thoracic ducts were cannulated, or the mice were killed and the spleens removed. The thoracic duct lymph from such mice contains less than 0·5% B cells (Basten *et al.*, 1972).

Adult thymectomized and reconstituted mice

CBA mice were thymectomized at 6–8 weeks, and irradiated (850 rad) 2–4 weeks later. They were then injected intravenously with 5×10^6 syngeneic fetal liver cells, and kept 1–2 months before use. Any animal showing thymic remnants at autopsy was discarded.

Capping of surface immunoglobulin

Fluorescein conjugated purified sheep antibody to rabbit IgG was a generous gift of Dr P. W. Kincade. Sheep antibodies to rabbit IgG (DEAE fraction 0·01 M tris—HCl pH 8·0 —DE 52 cellulose) were passed over a sepharose immunoadsorbent of normal mouse

globulin to remove cross-reactive antibodies. Antibodies were eluted from an immunoadsorbent of the rabbit IgG and fluorescein labelled (Kincade & Cooper, 1971). The F/P ratio was 2·8 and the antibodies were used at 0·5 mg/ml. Fluorescent cells were detected using a Leitz Orthoplan microscope, with type 250 lamp housing, 200 W ultra high pressure mercury lamp, BG 38 red suppression filter, 2 × KP490 exciting filter, TK 510 dichroic beam splitting mirror and K515 suppressor filter.

Ten million A.TL spleen cells were held at 4°C for 30 min with a 1 : 30 dilution of rabbit anti mouse IgM serum. They were then washed, and held for 15 min with fluorescein conjugated sheep anti rabbit IgG, washed again, and warmed to 37°C for 10 min. An aliquot of cells was removed and examined for capping by fluorescence microscopy. The remaining cells were cooled to 4°C, held with ^{125}I anti Ia serum or normal A.TH serum, and processed for radioautography as previously.

Characterization of Ia antigens

A.TL spleen cells were treated with tris buffered ammonium chloride to remove red cells (Boyle, 1968), and then dead cells were removed as previously. Fifty million cells were surface iodinated with ^{125}I by the lactoperoxidase technique (Marchalonis *et al.*, 1971) using a total of 2·5 mCi ^{125}I-iodide. The cells were washed once, and lysed with 3·5 ml 1 % Nonidet P-40 (British Drug Houses, Melbourne) in phosphate buffered saline (PBS) pH 7·4 for 30 min at room temperature. The lysate was then centrifuged at 1000 ***g*** to remove debris, and dialysed overnight at 4°C against PBS. It was then centrifuged at 10,000 ***g*** for 30 min. The supernatant was passed over a small column of rabbit anti mouse IgG serum coupled to Sepharose 4B (immunoglobulin binding capacity 400 μg/ml gel). The effluent was then held at 37°C for 1 hr with 500 μl of 0·1 mg/ml gamma globulin fraction of A.TH anti A.TL serum or normal A.TH serum. Mouse immunoglobulin was then quantitatively precipitated with rabbit anti mouse immunoglobulin. The precipitates were washed four times, and then boiled in a buffer solution containing 5% mercaptoethanol and 3% sodium dodecyl sulphate, and analysed by discontinuous polyacrylamide gel electrophoresis as described by Laemmli & Favre (1973). The total concentration of polyacrylamide was 10%, and the concentration of bis acrylamide was 0·25%. Gels were sliced and counted in a Packard gamma counter. Calibration of molecular weights was performed by running parallel gel electrophoresis of proteins of known molecular weight.

RESULTS

Establishment of conditions

A number of preliminary experiments were performed to establish suitable conditions for radioautography. It was found that a suitable degree of labelling was achieved by a concentration of ^{125}I A.TH anti A.TL serum of 25 μg/ml, and an exposure time of 3 days. Under these conditions, it was clear that a subpopulation of lymphocytes in spleen and lymph node were very heavily labelled, while the remainder were not significantly labelled above background.

A very clear visual distinction between heavily labelled and lightly labelled cells was observed over a concentration range of 15–50 μg/ml of antiserum, and exposure times of 2–10 days. An indication of the distribution of grains is shown in Fig. 1 (3 day exposure, 25 μg/ml). It should be noted that visually the distinction between lightly labelled and

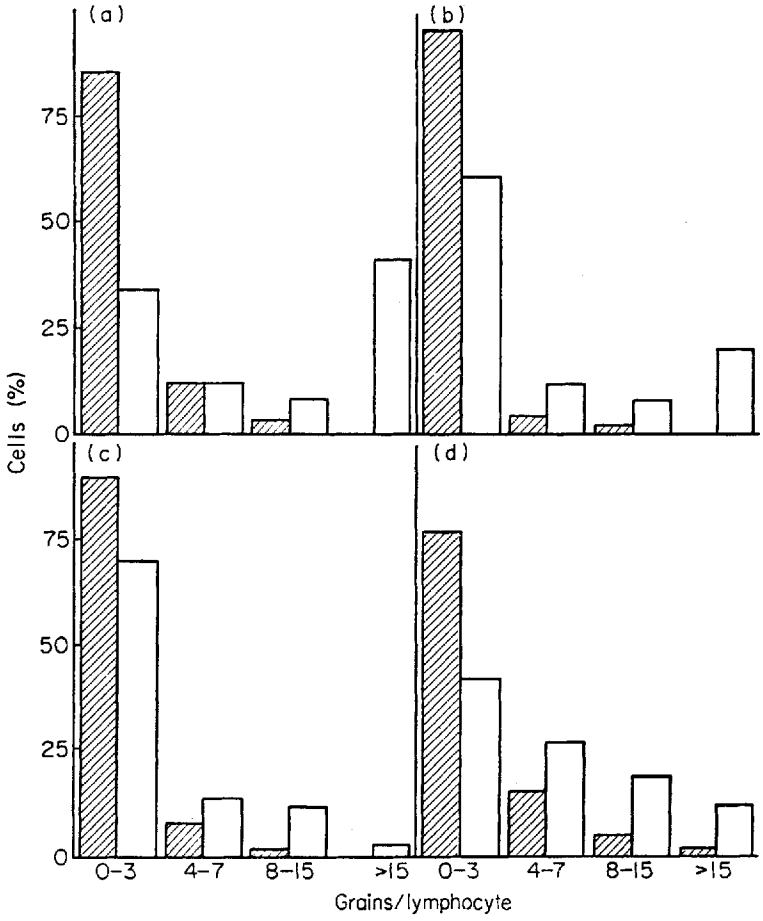

FIG. 1. Grain counts after treatment of A.TH (shaded) or A.TL (clear) cells with 25 μg/ml ^{125}I A.TH anti A.TL serum. Exposure time was 3 days. (a) Spleen; (b) lymph node; (c) thymus; (d) bone marrow.

heavily labelled cells was much more striking than would appear from the histograms, because most of the cells in the category of greater than fifteen grains were so heavily labelled that accurate grain counts were impossible (50–100 grains/cell). Except where noted, all subsequent experiments were performed using an antiserum concentration of 25 μg/ml and an exposure time of 3 days.

Control smears (binding of A.TH anti A.TL serum to A.TH cells) showed in general a very low degree of labelling. However, in early experiments significant but light labelling was noted in A.TH thymocytes, but not A.TH lymph node cells. This labelling was apparently due to autoantibodies directed against thymocytes, as it could be completely abolished by absorption with A.TH thymocytes. In subsequent experiments, antisera were routinely absorbed for autoantibodies. After such absorptions, control smears were very satisfactory in thymus and lymph node, although some persistent non-specific labelling remained in spleen and bone marrow (Fig. 1).

While there was a striking bimodal distribution of grains in lymph node and spleen, the distribution of grains was very heterogeneous in both thymus and bone marrow, with no indication of discrete binding subpopulations in these organs. It was consistently noted that there was a higher percentage of heavily labelled cells in spleen than in lymph node. After absorption of autoantibodies there was persistent specific, though very light, labelling in thymus. In a second experiment (Table 1), A.TH anti A.TL serum was absorbed sequentially with two aliquots of A.TH thymocytes, and longer exposure times used. Highly significant labelling of A.TL thymocytes was observed.

TABLE 1. Grain counts after 10-day exposure of A.TH and A.TL thymocytes to 25 μg/ml ^{125}I A.TH anti A.TL serum. Paired differences were tested for significance by Student's *t*-test; $P < 0{\cdot}001$

Serum	Cells	Grains/cell
^{125}I A.TH anti A.TL	A.TH thymocytes	$1{\cdot}02 \pm 0{\cdot}18$
^{125}I A.TH anti A.TL	A.TL thymocytes	$6{\cdot}89 \pm 0{\cdot}93$

Use of non-congenic mice as targets for A.TH anti A.TL serum

Because of a shortage of the congenic mice (they appear to breed very poorly), and in order to allow experiments involving non-congenic mice, A.TH anti A.TL serum was tested for binding to cells from CBA mice. These mice possess the H-2^k major histocompatibility complex, and hence the k haplotype in the I region. Provided that there are only significant antibodies against the I region antigens in A.TH anti A.TL serum, CBA mice should be a valid target for such antisera. Experiments of this type were highly satisfactory. The bimodal distribution of labelling was well preserved, and there was no evidence of significant natural antibodies to CBA antigens in unimmunized A.TH serum.

Nature of the heavily labelled cells

A variety of subsequent experiments was performed to determine the nature of the heavily labelled cells. The consistent finding of more heavily labelled cells in spleen than in lymph node, and the considerable number of heavily labelled cells in bone marrow but not in thymus, strongly suggested that the heavily labelled cells were B lymphocytes. When a population of A.TL lymph node cells were treated with anti Thy 1.2 serum and complement, an increase in the fraction of heavily labelled cells was consistently observed. In one experiment, for example, the percentage of cells with more than seven grains increased from 31% to 98%, while the percentage of cells bearing surface immunoglobulin increased from 29% to 96%.

When adult CBA thymectomized, irradiated and fetal liver reconstituted mice were used as targets, there was a similar increase (Table 2), which was more pronounced in lymph node than in spleen. Similarly, when A.TH anti A.TL serum was tested for binding to cells from CBA nude mice, the great majority of spleen cells showed heavy labelling (Table 3). Approximately 75% of spleen cells from these mice have high density surface immunoglobulin (Osmond & Nossal, 1974).

A.TL spleen cells were fractionated by cell electrophoresis, and each fraction tested for binding of A.TH anti A.TL serum or anti IgM serum. The results are shown in Fig. 2. The

TABLE 2. Percentage cells binding A.TH anti A.TL serum in normal CBA mice and CBA mice depleted of T cells. The concentration of antiserum was 25 μg/ml, and the exposure time was 3 days. Cells with more than seven grains were scored as positive

Organ	Labelled cells (%)	
	Normal CBA	T cell depleted
Spleen	55	78
Lymph node	25	89

TABLE 3. Binding of ^{125}I A.TH anti A.TL serum to spleen cells of congenitally athymic ('nude') mice bearing the H-2^k chromosome. Conditions as for Fig. 3. Mean ± standard error of three experiments

Serum	Labelled cells (%)
A.TH anti A.TL	78·7 ± 5·2
Normal A.TH	1·0 ± 0·3

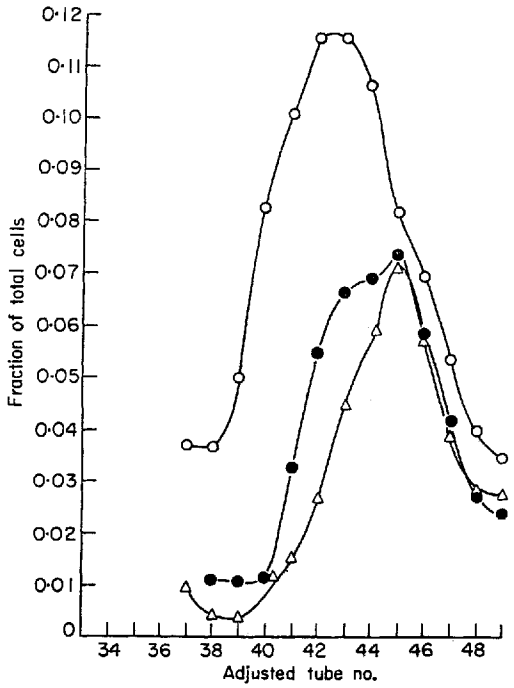

FIG. 2. Analysis of relative electrophoretic mobility of cells bearing Ia determinants and cells bearing high density surface immunoglobulin. ○, Total cells (Coulter counter); △, Ig+; ●, Ia + (A.TH anti A.TL).

Ig+ and Ia+ peaks overlap almost completely, although there was a slight excess of 'Ia+, Ig–' cells on the left of the main binding peak. These cells could represent either labelled T cells or 'null' (Thy 1.2 negative, Ig–) cells. It is of interest that it is in precisely this position that the null cells are found (von Boehmer, 1974), and that by adding the 'null' and 'Ig+' figures, a curve very similar to the 'Ia+' curve can be constructed.

On the other hand, cells from populations strongly enriched for T cells showed greatly reduced labelling. The binding of radio-iodinated A.TH anti A.TL serum to CBA thymocytes activated to C57Bl histocompatibility antigens is shown in Table 4. There was absolutely no detectable labelling of thoracic duct lymphocytes (T.TDL's) from these animals. A very slight degree of labelling in spleen cells (splenic ATC's) was noted.

TABLE 4. Binding of ^{125}I A.TH anti A.TL or normal A.TH serum to CBA thymocytes activated to C57Bl histocompatibility antigens. Paired differences were tested for significance by Student's *t*-test. Spleen cells showed marginally significant labelling ($P = 0{\cdot}05$), while the differences for thoracic duct lymphocytes were not significant. Conditions as for Fig. 1

Serum	Cells	Grains/cell		
		0–3	4–7	>7
A.TH anti A.TL	Spleen	77	13	10
Normal A.TH	Spleen	83	14	3
A.TH anti A.TL	Thoracic duct	93	7	0
Normal A.TH	Thoracic duct	97	3	0

Ia antigens on plaque forming cells

Initial experiments to test for the presence of Ia antigens on plaque forming cells (PFC) gave negative results. When 50×10^6 immune CBA spleen cells were incubated with 200 μl 1:10 A.TH anti A.TL serum, washed, and treated with complement, a reduction of only 10–15% in PFC was noted. However, when the cell numbers were reduced to 10^7, and undiluted anti Ia serum was used, a very marked reduction in PFC was observed (Table 5).

TABLE 5. Effect of anti Ia serum and complement on CBA plaque forming cells. Results are expressed as a percentage of untreated cells (mean ± standard error; three replicates per group)

Treatment	Plaque forming cells after treatment (%)	
	Direct	Indirect
Complement alone	100	100
A.TL anti A.TH + C′	90 ± 8·5	88 ± 4·8
A.TH anti A.TL + C′	6 ± 0·6	6 ± 0·9
Balb/c anti CBA + C′	3 ± 0·7	1 ± 0·9

This reduction was specific, since A.TL anti A.TH (anti I^s) serum caused no significant reduction of plaque numbers.

Occasional cells having the morphological characteristics of plasma cells in spleen smears appeared to be very lightly labelled. An H-2^k plasma cell tumour C1.18 derived from C3H mice, was also only lightly labelled. After 10 days exposure, there were 14·9 ± 1·8 grains/cell when A.TH anti A.TL serum was used, and 11·2 ± 1·4 grains/cell with A.TL anti A.TH. The differences were not statistically significant. A second experiment gave similar results. Cells from this tumour lack an Fc receptor (N. L. Warner, unpublished data).

Ia antigens on T cell lymphomas

We have also examined a series of some ten spontaneous lymphomas in A.TH, A.TL and A.SW mice for Ia antigens. All of these tumours have been shown to be positive for Thy 1.2 antigen and negative for surface immunoglobulin by immunofluorescence, and are hence of T cell origin. In every case, the tumours were rejected when transplanted into mice differing at the I region, but only very weak binding was demonstrable with anti Ia serum. Rejection was not due to incompatibility at the TL locus. For example, A.TL tumours were rejected when transplanted into A.TH mice, while they grew rapidly in syngeneic mice. They were also rejected when transplanted into A.TH × B10.D2 F_1 hybrids, indicating that the rejection was not due to TL incompatibility (Goding & Warner, 1975; see discussion section for details of TL differences between A.TH and A.TL mice).

Independent movement of surface immunoglobulin and Ia antigens

In view of the association between the I region and specific immune response genes, it was of interest to determine the relationship between Ia antigens and B cell surface immunoglobulin. If Ia antigens were immunoglobulin in nature, or were physically associated with immunoglobulin in the membrane, it might be expected that pretreatment of cells with anti immunoglobulin would inhibit the binding of anti Ia serum to B cells and/or that 'capping' of B cell surface immunoglobulin would result in capping of Ia antigens. The results of such an experiment are shown in Table 6. It is clear that there was no significant reduction in the percentage of labelled cells following pretreatment with anti immunoglobulin, and that capping of surface immunoglobulin did not lead to capping of Ia antigens. Similar results

TABLE 6. Independent capping of cell surface immunoglobulin and Ia antigens. Anti Ia serum was used at a concentration of 25 μg/ml, and exposure time was 3 days. Cells having more than seven grains were scored as positive

Cells	Labelled (%)	Capped labelled cells (%)
A.TL spleen cells labelled with FITC anti-immunoglobulin (sandwich) under capping conditions	45	91 (fluorescence)
As above, then cooled to 4°C and held with ^{125}I A.TH anti A.TL serum	61	2 (radioautography)
A.TL spleen cells held with ^{125}I A.TH anti A.TL serum at 4°C; no prior treatment	60	3 (radioautography)
A.TL spleen cells held at 4°C with ^{125}I A.TH anti A.TL, washed, held with rabbit anti-mouse Ig under capping conditions	54	75 (radioautography)

were obtained when the Fc receptor on B cells was capped prior to treatment with anti Ia serum (J. F. A. P. Miller & J. W. Goding, unpublished data).

To determine whether Ia antigens on B cells could be capped, spleen cells from A.TL mice were held at 4°C for 30 min with ^{125}I A.TH anti A.TL washed, and then held with a strong polyvalent rabbit anti mouse IgG serum for 30 min at 4°C. The cells were washed again, warmed to 37°C for 15 min, and processed for autoradiography. Following this procedure, about three-quarters of the labelled cells were capped as assessed by polar distribution of grains (Table 6).

Characterization of Ia antigens

In order to characterize Ia antigens, spleen cells from A.TL mice were surface radioiodinated with ^{125}I using the lactoperoxidase technique, lysed with NP-40, and the products precipitated with A.TH anti A.TL serum and analysed by polyacrylamide gel electrophoresis under reducing conditions in the presence of sodium dodecyl sulphate (Fig. 3). A major peak, of molecular weight approximately 30,000, which migrated just behind the light chain

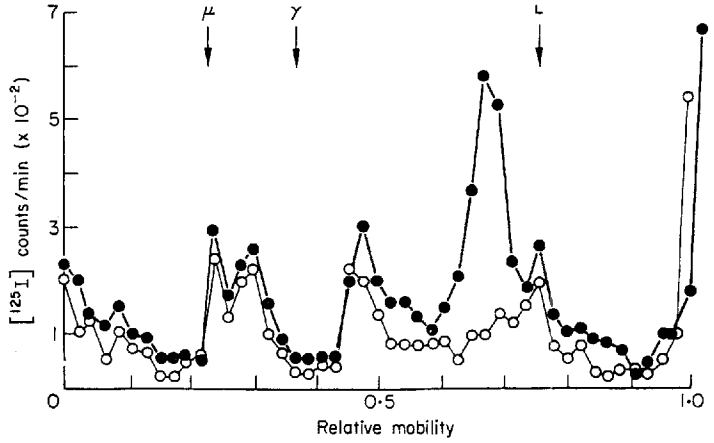

FIG. 3. Analysis by SDS polyacylamide gel electrophoresis of products precipitated from detergent lysates of ^{125}I surface labelled A.TL spleen cells by A.TH anti A.TL serum (–●–●–), or normal A.TH serum (–○–○–).

peak, was observed in the experimental (A.TH anti A.TL) group, but not in the control group (normal A.TH serum). Depletion of radiolabelled surface immunoglobulin was not absolute, and small peaks are seen corresponding in mobility to μ and light chain markers, in both experimental and control gels.

As in other work (Melcher *et al.*, 1974; Hunt & Marchalonis, 1974; Haustein & Goding, submitted), two heavy chain peaks were observed. These might differ in carbohydrate (Hunt & Marchalonis, 1974), or might represent distinct classes of B cell surface immunoglobulin (Melcher *et al.*, 1974). Another minor peak, which migrated slightly ahead of the γ chain standard, was also present. This component has been observed elsewhere, and it has been shown to possess an affinity for antigen–antibody complexes (Haustein, Marchalonis & Harris, 1975).

DISCUSSION

Mouse antibodies appear to be particularly susceptible to denaturation by conventional methods of coupling of fluorochromes, 2,4-dinitrophenyl, ferritin, peroxidase and radio-iodination by non-enzyme methods (Hämmerling *et al.*, 1974). The results show that lacto-peroxidase catalysed ^{125}I radio-iodinated anti Ia antisera are a useful tool in the study of Ia antigens.

That the labelling of cells was specific is shown in Fig. 1, which demonstrates that only 2–4% of A.TH spleen or lymph node cells were significantly labelled by A.TH anti A.TL serum. Thus, significant binding to lymphocytes via Fc receptors because of aggregates or complexes was ruled out, as was significant labelling due to autoantibodies. Problems due to non complement fixing antibodies, and variations in complement potency and toxicity, which plague cytotoxicity testing, are thus circumvented. However, the technique depends on having antisera of high titre (the A.TH anti A.TL serum used had a titre of 1:2000 by semi-micro cytotoxicity methods), and has so far not proven useful with weaker mouse alloantisera (unpublished data).

The results leave no doubt that the heavily labelled cells were B cells. This is indicated by the organ distribution, effects of anti Thy 1.2 serum, the results of cell population enriched and depleted for B cells, and information from cell electrophoresis. The data are consistent with those of Hämmerling *et al.* (1974), and Sachs & Cone (1973). It would appear that rather more cells bind anti Ia serum than bind anti Ig serum. This was particularly noticeable in the spleen, where a small number of cells (perhaps 5–10%) bound anti Ia but not anti Ig serum (Fig. 2, Table 6).

Our results also confirm those of Hämmerling *et al.* (1974), who showed an 84% reduction in plaque forming cells from C3H (H-2^k) mice treated with A.TH anti A.TL serum and complement. These authors did not specify the dilution of serum used, and only direct (IgM) plaques were studied. Frelinger *et al.* (1974b) were only able to partially (50%) inhibit plaque forming cells with anti Ia serum, when using a 1:100 dilution. In view of the very high cytotoxic titre of these antisera against spleen and lymph node cells, these results would suggest that Ia antigens are present in much smaller amounts on plaque forming cells than on other B cells. Such a notion is supported by our finding that an H-2^k plasma cell tumour (C 1.18) was not significantly labelled with anti Ia serum.

On the other hand, our results provide strong evidence for the presence of Ia antigens on T cells. This is most clearly indicated in thymus, where rather heterogeneous light but specific labelling was demonstrated (Fig. 1, Table 1). The overall grain counts could not be explained on the basis of B cell contamination, as the labelled cells in the thymus were far more numerous than B cells (approximately 0·5% of thymocytes bore high density surface immunoglobulin). The labelled cells were much more lightly labelled than B cells.

A.TH and A.TL mice differ at the TL locus as well as at the I region (Frelinger *et al.*, 1974a). This is the only known antigenic difference outside the H-2 segment. A.TH is Tla^a (+1, 2, 3) while A.TL is Tla^c (+2). These differences arose because the D end of A.TH was derived from H-2^a while the D end of A.TL was derived from H-2^d. The H-2^a chromosome is thought to have arisen by crossing over between H-2^k and H-2^d, but it is not entirely clear why there should be a Tla difference. A mutation or second crossover early in the development of the H-2^a chromosome is possible. It is clear that A.TL carries no Tla specificities that are not also carried by A.TH, and thus anti-Tla antibodies would not be expected in this

direction. Moreover, Tl antigens are not (as far as is known) expressed on spleen cells, yet the labelling in thymus can be totally abolished by absorption with A.TL spleen cells (unpublished data). It is, therefore, considered that the T-cell binding observed in the thymus was due to anti Ia antibodies.

The presence of Ia antigens on T cells is also strongly suggested by the consistent rejection of many different T cell tumours when transplanted into mice which were incompatible at the I region. In at least one instance, such rejection has been shown to be associated with IB or IC region incompatibility (Goding & Warner, 1975).

The presence of Ia antigens on T cells has also been reported by Götze *et al.* (1973), but in this publication there was, surprisingly, no evidence for Ia antigens on B cells. Other evidence for Ia antigens on T cells comes from Frelinger *et al.* (1974b), who were able to show their presence using an extremely sensitive microcytotoxicity test, and Lonai & McDevitt (1974) who showed that in some situations (especially IC incompatibility) T cells were able to stimulate in the mixed lymphocyte reaction.

The structural genes for mouse immunoglobulin heavy chains (as shown by heavy chain allotypic markers) are not linked to H-2. However, the location in the genome of the genes coding for the K light chains in the mouse is not known (Cohn, 1972), although in man the InV (light chain) allotypes are not linked to HL-A (Gally & Edelman, 1972). It was thus possible that the Ia antigens might have represented polymorphisms of K light chains, which in turn relate to *Ir* gene effects. However, the demonstration (Table 6) that B cell surface immunoglobulin can be 'capped' without altering the distribution of Ia antigens shows clearly that these surface components consist of different molecules. Our data do not exclude the possibility that a minor subcomponent of Ia antigens is immunoglobulin.

Other strong evidence indicating that the Ia antigens are not immunoglobulin comes from the isolation data. A significant cell surface component precipitable with anti Ia serum is seen on polyacrylamide gel electrophoresis (Fig. 3) following depletion of immunoglobulin. This component has a different mobility on polyacrylamide gel electrophoresis to that of any heavy or light chain described.

It is of interest to note that under strong capping conditions (Table 6) only 75% of cells bearing Ia antigens could be capped. The inability to cap the remaining 25% could have at least two reasons. Firstly, if caps are in the axis of the light path, they may not be recognized in smears. Alternatively, it may be that the uncapped cells have a rather low surface density of Ia determinants, preventing their efficient cross-linking.

Analysis of ^{125}I cell surface material precipitated by anti Ia serum revealed a single specific peak of molecular weight approximately 30,000 as determined by polyacrylamide gel electrophoresis. This is in general agreement with the results of Cullen *et al.* (1974), Vitetta *et al.* (1974) and McDevitt *et al.* (1974). Our gel patterns show a relatively sharp peak, suggesting limited heterogeneity with regard to overall molecular weight, although the work of Cullen *et al.* (1974) has shown that at least two antigenically distinct molecules can be precipitated by anti Ia sera in a similar system.

It has been proposed (Dickler & Sachs, 1974) that the Ia antigens represent polymorphisms of the Fc receptor on B cells. This proposal was based on the finding that anti Ia serum could block binding of aggregates and complexes to the Fc receptor. We do not favour such an hypothesis, for the following reasons. Firstly, the data presented showed that anti Ia serum directed at *either* parental I region of F_1 B cells were potent (90%) inhibitors of binding to Fc receptors. These results suggest that the observed blocking was due to steric

hindrance, since one would expect that if Ia antigens were the Fc receptor, a maximum of 50% inhibition would be seen for anti Ia directed against either parental antigen of an F_1 hybrid. Secondly, work has shown that Ia antigens and the Fc receptor do not co-cap (J. F. A. P. Miller, unpublished data; B. Pernis, personal communication). As for surface immunoglobulin, the possibility that a *subclass* of Ia antigens might be the Fc receptor has not been entirely excluded. Furthermore, if any of the Ia antigens had the properties of the Fc receptor, they should be precipitable with *any* immune complex. Recent work (Haustein, Marchalonis & Harris, 1975) suggests that the radio-iodinated component which migrates slightly faster than the γ chain is a possible candidate for the Fc receptor. This component was precipitated by a variety of antigen–antibody complexes.

The functional expression of the H-2 linked *Ir* genes in T cells suggested that the Ia antigens would also be expressed in T cells. It has been proposed (Benacerraf & McDevitt, 1972) that the Ir-1 gene codes for the T cell receptor for antigen, a non-immunoglobulin molecule. Our results show clearly that Ia antigens are expressed predominantly on B cells, but the data indicate weaker though unequivocal expression in at least some T cells. The data lend no support to the notion that the Ir-1 gene product is the T cell receptor, but neither do they disprove it. If the T cell products coded for by this gene were *clonally distributed*, they might not be detected by our approaches.

There is now considerable evidence against such an hypothesis, however. High and low responder T cells bind roughly equal amounts of (T,G)-A--L (Hämmerling & McDevitt, 1974a, b) and this binding is inhibitable by antisera directed at *both* the K (including I) and D (not including I) region antigens. Anti Ia serum does *not* inhibit antigen binding by T cells (Hämmerling & McDevitt, 1974c). Nabholz *et al.* (1974) found no inhibition of T cell mediated lympholysis by anti H-2 sera directed against the killer cells.

The inhibition of certain lymphocyte functions, such as antigen induced proliferation, by alloantisera in the guinea-pig is an often quoted argument in support of the receptor hypothesis. However, such inhibition follows the histocompatibility gene status rather than the responder status in animals where these have been separated (Shevach *et al.*, 1974). The inhibition is ineffective when Fab_1 fragments of alloantisera are used, even when the reduced avidity of monomeric fragments is taken into account. The inhibition is effective even if the cells are only exposed to the alloantisera several hours after contact with antigen (Bluestein, 1974).

What, then, are the functions of the Ia antigens? How can the *Ir* gene effects be related to them? At present these questions cannot be firmly answered.

It is clear that the Ia antigens are heterogeneous. At least three I regions and ten Ia specificities have been described (Shreffler & David, 1974). Future work will have to concentrate on dissecting this heterogeneity, as the antigens controlled by the various regions may have different functions. We propose that the heavy labelling of B cells compared to T cells, and the suggestive evidence of much smaller amounts of Ia antigen on plaque forming cells than on other B cells, indicate that at least one Ia antigen is a differentiation antigen on mature virgin and memory B cells.

An attractive hypothesis is that the B cell Ia antigens represent a receptor site for the T cell 'second signal' needed for B cell activation (Katz & Benacerraf, 1974). The recent demonstration of Ia determinants on 'allogeneic effect factor' (Armerding *et al.*, 1974), the apparent need for I-region identity for T–B collaboration (Katz *et al.*, 1974), and the experiments of Taussig (1974a, b, c) showing an antigen specific T cell collaborative factor

with H-2 determinants may represent clues to *Ir* gene function. In the latter instance, evidence was presented that T cells from both responders and non-responders produce the collaborative factor, but only *responder* B cells are capable of activation by it. Thus, the defect in the non-responders is at the B cell level. On the other hand, this model does not account for the clear demonstration that only responders can mount delayed type hypersensitivity (an exclusive T cell function) against antigens the response to which is under histocompatibility-linked *Ir* gene control (Green *et al.*, 1966). Thus, the block in the non-responders could be at any step in the activation of the T cell, but the *specificity* of the *Ir* gene effects remains unexplained.

Work is in progress to explore relationships between functional *Ir* genes and the Ia antigens.

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