

EVIDENCE FOR THE EXPRESSION OF Ia  
(H-2-ASSOCIATED) ANTIGENS ON  
THYMUS-DERIVED LYMPHOCYTES\*

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The *H-2* gene complex of the mouse can be divided into four regions, K, I, S, and D (1, 2). The K and D regions control the classical *H-2* antigens which are detected serologically by serum antibodies, and also by graft rejection. The S region contains the genes which regulate the Ss and SIp protein variants in the serum (3). The I region contains immune response (*Ir*) genes, which regulate the antibody responses to many antigens, both natural and synthetic (4).

It has recently been shown that genes responsible for the strongest reactions in graft-vs.-host (5) and mixed leukocyte reaction (MLR)<sup>1</sup> (6, 7) map in the I region; weaker MLR reactions can be caused by the K or D regions alone. Differences in the I region can also result in graft rejection (8; Shreffler, unpublished observations).

Recently, we have described an antiserum directed against antigens mapping in the I region of the *H-2* complex (9-11). These antigens originally named Lna (9), are now designated Ia (I region-associated antigens) (32). Similar antigen systems also mapping in this region have been described by others (12-14). *Ir* genes are thought to be expressed primarily in T cells (4, 16), although effects in B cells can be demonstrated (17-19). If Ia antigens were related to *Ir* products they would be expected to be expressed on T cells, but also perhaps on B cells. The original reports of David et al. (9), Hauptfeld et al. (12), and Götze et al. (14) suggested that Ia antigens were expressed on T cells. Later reports by Sachs and Cone (13) and Hammerling et al. (15) reported that the antigens were expressed exclusively on B cells.

In this report, we describe further studies on the cellular distribution of antigens detected by our original antiserum, A.TH anti-A.TL, and evidence for specific reactions with T lymphocytes, as well as B lymphocytes, as detected by a sensitive microcytotoxic test.

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<sup>1</sup> Abbreviations used in this paper: MBLA, mouse-specific B lymphocyte antigen; MLR, mixed leukocyte reaction; PFC, plaque-forming cells.

## Materials and Methods

Mice were raised in our colony at the University of Michigan. A.TH mice are  $H-2^{12}$  ( $K^* I^* S^* D^d$ ) and A.TL mice are  $H-2^{11}$  ( $K^* I^* S^* D^d$ ) (9). BALB/cdJ ( $H-2^c$ ) mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. BALB/c nude mice were raised from N, nu/+ breeders which were a generous gift of Dr. E. A. Boyse. A.TH anti-A.TL antiserum was drawn from two large pools prepared as previously described (9). Anti-Thy-1<sup>b</sup> was prepared by immunization of AKR mice with C3H thymocytes according to Reif and Allen (20) (50% titer with C3H thymus cells = 1/2,560). Mouse-specific B lymphocyte antigen (MBLA) was prepared as previously described by Niederhuber (21) (50% titer with bone marrow cells 1/32). Dye exclusion microcytotoxic tests were done as previously described (9), except that nigrosin was substituted for trypan blue for the visualization of dead cells, and the cell number in each well was decreased to 700.

In vivo absorptions were performed as previously described (22). In vitro absorptions were done by adding 100  $\mu$ l of appropriately diluted antiserum to a counted number of packed absorbing cells. After 1 h at room temperature, the suspension was centrifuged and the supernate tested for residual antibody activity. For absorptions using thymocytes, care was taken to exclude the parathymic lymph nodes.

To test the effect of anti-Ia serum on plaque-forming cells (PFC), mouse spleen cells primed with sheep red blood cells (SRBC) were obtained by injection of  $4 \times 10^8$  SRBC 6 days before sacrifice. This preparation was treated with anti-Thy-1<sup>b</sup>, and two concentrations of anti-Ia sera in the presence of complement. The remaining cells were plaqued with SRBC by the method of Jerne (23).

To determine if anti-Ia, anti-Thy-1, and anti-MBLA were reacting with different cell populations, equal volumes of each of the appropriate antisera (Ia, Thy-1, MBLA) were mixed in such a manner that the concentration of each antiserum was equivalent to that used in the unmixed sera. These mixed sera were assayed for their cytotoxic effects on normal cell populations. T cells were purified on nylon wool columns by the method of Julius et al. (24). Cortisone-resistant thymocytes were prepared by intraperitoneal injection of 10 mg cortisone, 48 h before sacrifice (25). These cells are thought to be a more mature population of T cells than normal thymocytes. A C57Br( $H-2^k$ ) Gross virus-induced thymic leukemia L251A, grown in tissue culture, was supplied by Dr. Ronald T. Acton, University of Alabama, Birmingham, Ala. (33).

## Results

*Assay.* The cytotoxic test in use in our laboratory has been modified in several ways to increase its sensitivity. The use of medium 199 in place of Hanks' balanced salt solution which, we have found, allows the use of higher concentrations of rabbit complement, along with a two-stage test in which the complement incubation is done in the absence of fetal calf serum, have together increased the sensitivity of this test to an extremely high level. Many of the reactions described are not detectable using either a <sup>51</sup>Cr-release or a standard semimicro dye exclusion test done in tubes.

*Direct Cytotoxic Test.* We had previously reported, using A.TL or A.TH lymph node lymphocytes at low antiserum concentration (dilutions of 1/500 or greater), a maximum cytotoxicity of 50%. As can be seen in Fig. 1, the maximum kill at high serum concentration with A.TL lymph node cells is 70%, but a distinct and reproducible biphasic titration curve is seen, the titer of the first break being approximately 1/100. Beyond this point, the level of killing of approximately 50% persists to the end point of the titration. Direct tests with splenic lymphocytes (Fig. 2) also show a similar biphasic titration curve, but generally a lower maximum percentage kill (50–60%) and a lower plateau of killing (20–30%) than lymph node lymphocytes. Thymus cell targets act in a

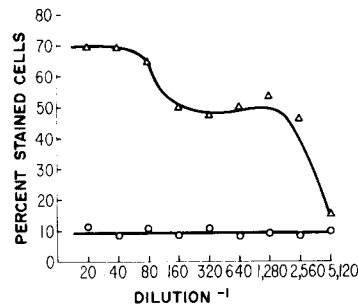


FIG. 1. Direct cytotoxic test of A.TH anti A.TL ( $\Delta$ — $\Delta$ ) tested with A.TL lymph node target cells; ATH normal serum (O—O).

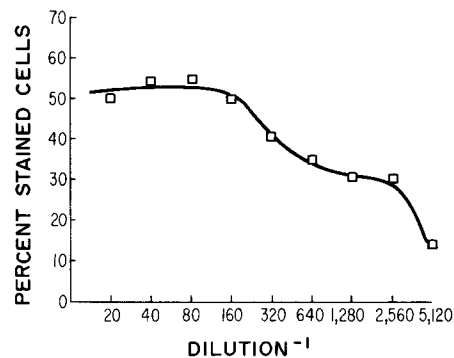


FIG. 2. Direct cytotoxic test of A.TH anti A.TL tested with A.TL splenic lymphocytes.

weak, irregularly reproducible manner, in direct tests, occasionally resulting in up to 20% killing (10% above baseline) with titers up to 1/1,000.

Cortisone-resistant thymocytes, which were greater than 95% sensitive to anti-Thy-1<sup>b</sup> serum, when tested with anti-Ia serum gave killing in the range of 30–50%, with titers comparable to lymph node and spleen cells (Fig. 3). However, these cells fail to demonstrate the pronounced biphasic titration curve observed with lymph node and spleen cell targets. When T cells were purified on nylon wool columns (Leukopak, Fenwal Laboratories, Morton Grove, Ill.), enrichment of Thy-1<sup>b</sup>-positive cells from spleen from 30% up to 80% was obtained. From lymph nodes a more modest enrichment of T cells from 65 to about 75–80% was achieved. The yield of Thy-1<sup>b</sup>-positive cells from spleen was about 80–90%, while from lymph nodes it was only 40–50%. These values are comparable to those reported by Julius (24). Cortisone-resistant thymus cells were also passed through the column to ascertain the effects of the column on a relatively pure (95%) T-cell population. When purified T cells from these three sources were tested in the cytotoxic test with the A.TH anti-A.TL serum, none were reactive, including the column purified cortisone-resistant thymocytes.

In most of these experiments, it was possible to account for the loss of Ia-positive cells by simple retention on the column. In other experiments it was necessary to postulate a conversion from Ia-positive to Ia-negative after passage

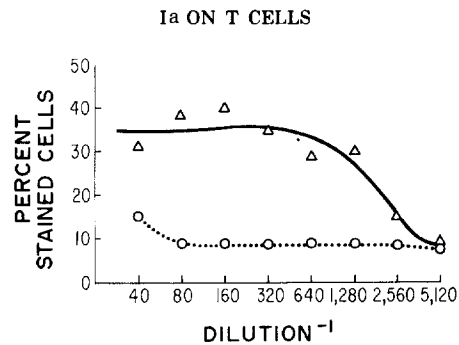


FIG. 3. Direct cytotoxic test of A.TH anti A.TL ( $\Delta$ - $\Delta$ ) tested with A.TL cortisone-resistant thymus cells; ATH normal serum (O-O).

through the nylon wool column. For example, in one experiment  $1.8 \times 10^7$  Ia-positive lymph node cells in a total of  $2.6 \times 10^7$  cells were placed on the column. As a maximum estimate,  $3 \times 10^6$  Ia-positive cells were recovered in a total of  $1.6 \times 10^7$  cells recovered. This leaves  $1.5 \times 10^7$  Ia-positive cells to be accounted for, but only  $10^7$  cells were retained on the column. This implies a minimum conversion of  $5 \times 10^6$  Ia-positive cells to Ia-negative. This does not seem to be a function of simple incubation of the cells in median, because cells held for similar lengths of time in tissue culture median alone do not lose expression of Ia antigens.

*Expression of Ia Antigens on Tumors.* Several tumors, both T- and B-cell-derived, and several tumor cell lines have been screened for reactivity with anti-Ia sera. These include L 251A (C57Br/cdJ,  $H-2^k$ ), H 111 (AKR/J,  $H-2^k$ ) HA4995 (AKR/J,  $H-2^k$ ), EL-4 (C57BL/10,  $H-2^b$ ), IB (C58,  $H-2^k$ ), Meth A (BALB/cJ,  $H-2^d$ ). Only L 251A was positive. This is a Gross virus-induced lymphoma derived from strain C57Br/cdJ ( $H-2^k$ ). Both anti-Ia and anti-Thy-1 sera kill 100% of these cells (Fig. 4). This shows that at least some T-cell tumor lines do express both Ia and Thy-1 antigens.

*Effects of Anti-Ia Serum on PFC.* A.AL ( $K^k I^k S^k D^d$ ) cells are equivalent to A.TL cells in reactivity with A.TH anti-A.TL. Spleen cells from A.AL mice which had been immunized with SRBC were treated with normal rabbit serum as a complement source, anti-Thy-1, and anti-Ia 1/100 and 1/1,000. The cytotoxic index of each of these preparations is noted as follows: defining the cytotoxic index for the complement control as 0, the CI for anti-Thy-1 was 27; anti-Ia 1/100, 55, and anti-Ia 1/1,000, 25. The residual concentration of PFC was determined after each treatment (Fig. 5). Anti-Thy-1<sup>b</sup>, and anti-Ia (1/1,000) gave no decrease in the PFC concentrations in spite of appreciable cytotoxicity. Anti-Ia at 1/100 did diminish the number of PFC by approximately 50%.

*Absorptions.* Repeated attempts to achieve specific absorption of A.TH anti-A.TL serum at concentrations greater than 1/100 were unsuccessful. With serum concentrations of 1/200, the serum was absorbed equally well by lymph node or spleen cells, but lymph node targets seemed to be generally slightly more sensitive to residual cytotoxic activity than spleen cells (Fig. 6 a and b). Normal thymus cells could also absorb this activity, but less effectively than either lymph node or splenic lymphocytes. Cortisone-resistant thymocytes were as

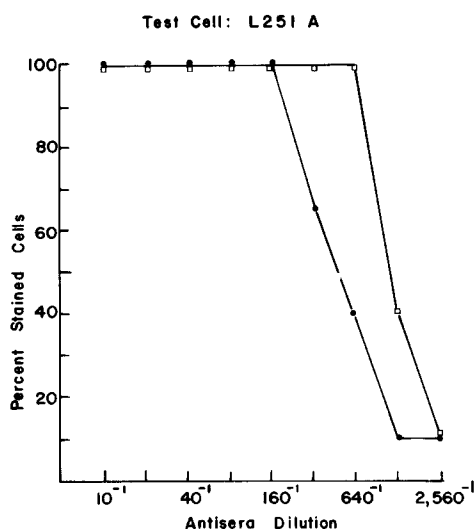


FIG. 4. Direct cytotoxic test of A.TH anti A.TL (●—●), and AKR anti C3H (□—□) tested with C57Br leukemic cell line L251A.

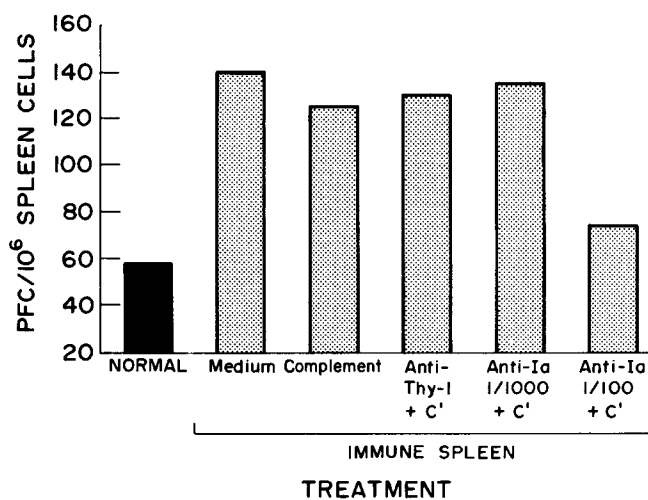


FIG. 5. Effect of anti-Ia serum on PFC. A.AL spleen cells primed in vivo with  $2 \times 10^8$  SRBC 5 days before testing. The normal bar represents unimmunized spleen.

effective in absorptions as spleen or lymph node cells. BALB/c-nu/nu mice (N<sub>7</sub>F<sub>1</sub>) were injected with undiluted A.TH anti-A.TL serum and bled after 2, 6, and 24 h. The resulting in vivo absorbed serum was tested for residual activity against nude and normal BALB/c lymph node cells and against cortisone-resistant BALB/c thymocytes. The resulting titration curve with normal lymph node targets is shown in Fig. 7. This serum was unreactive with nude cells, but contained residual activity against both normal BALB/c lymph node cells and cortisone-resistant thymocytes, both at 2 and 6 h after injection. After 24 to 30 h,

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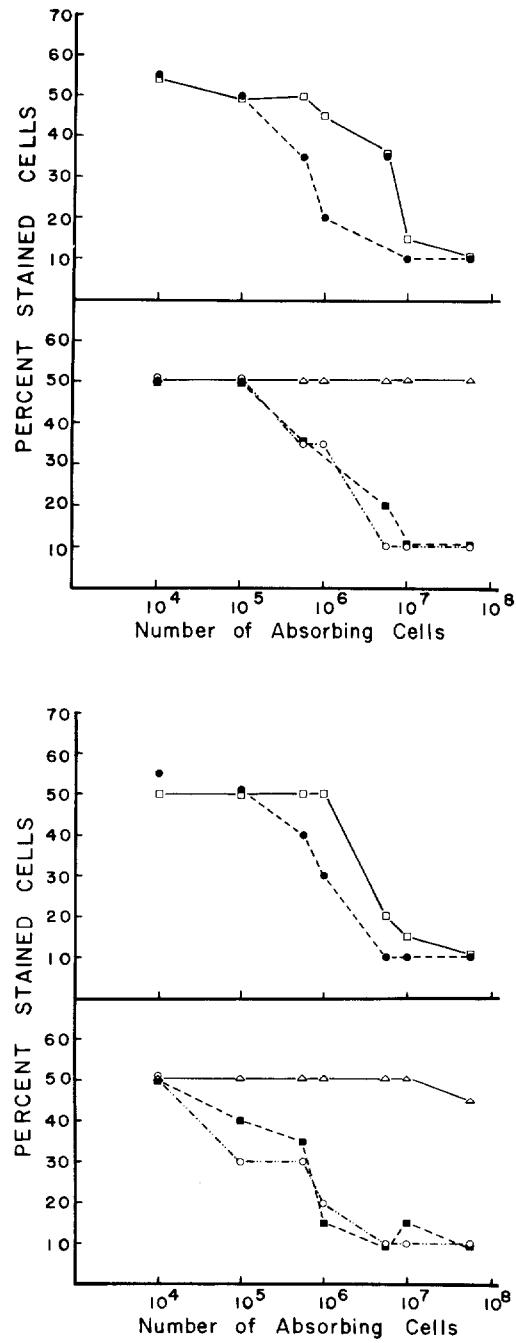


FIG. 6. (a) Absorption test of A.TH anti A.TL. Serum was absorbed with A.TH spleen cells (Δ-Δ). A.TL lymph node cells (O---O), A.TL spleen cells (■---■) (bottom), or A.TL thymus (□-□) or cortisone-resistant thymus (●---●) (top), and tested with A.TL lymph node lymphocyte targets. (b) Same sera tested with A.TL spleen cells.

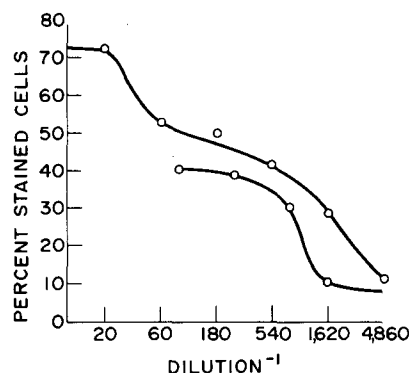


FIG. 7. In vivo absorption of A.TH anti A.TL in BALB/c nudes. Tested with normal BALB/c lymph node lymphocytes. Upper curve before absorption, lower after absorption. Plotted to correct for the dilution in vivo.

no detectable antibody remained, however. Normal BALB/c mice completely cleared equal amounts of anti-A.TL serum for all targets by 2-3 h.

*Mixed Antiserum Experiments.* These experiments were similar in design to those of Raff (26) to test for restriction of reactivity to a single class of cells. A.TH anti-A.TL serum was mixed with anti-Thy-1<sup>b</sup> serum or anti-MBLA serum and tested with A.TL lymph node targets. The effects of the mixed antisera were compared with the effects of each of the antisera tested separately on the same cell preparation. The results are shown in Table I. When anti-Thy-1 and anti-MBLA were mixed, all lymph node cells were killed. When low concentrations of anti-Ia (1/1,000) and Thy-1 were mixed, little additive killing resulted. However, when anti-MBLA and anti-Ia (1/1,000) were mixed, a marked increase in killing resulted. In order to test for possible interference by non-Thy-1 antibodies in the anti-Thy-1 serum, the reciprocal antiserum, C3H anti-AKR, was tested with A.AL, a Thy-1<sup>a</sup> strain, in similar mixing experiments with similar results. Similar experiments were also performed with ATL anti-ATH and anti-Thy-1<sup>b</sup> with similar results. In contrast, when similar experiments were performed using higher anti-Ia serum concentration additive killing results with both anti-MBLA and anti-Thy-1. In order to determine if some simple form of allelic exclusion was operating ( $A \times B10.S$ )F<sub>1</sub> lymph node cells were tested with anti-Ia<sup>s</sup> (A.TL- $\alpha$ -A.TH), anti-Ia<sup>k</sup> (A.TH- $\alpha$ -A.TL) and mixtures of the two antisera. If some simple form of allelic exclusion was operating then an increase in the toxicity of either antiserum alone would be expected. As shown in Table I, even the mixtures of the antisera do not show 100% killing. In fact there is no increase over the maximum killing of either antiserum alone. This provides evidence that Ia antigens are present only on a subset of lymphocytes and that no simple form of allelic exclusion is operating.

## Discussion

The central question we have been addressing is whether Ia antigens are

TABLE I  
*Relationship of Ia to Thy-1 and MBLA Antigens*

Antisera	Percent killing of target cells*:			
	A.TL LNC‡	A.AL LNC§	A.TH LNC	(A × B10.S)F <sub>1</sub> ‡
Anti-Thy-1 (1/32)**	60	60	65	65
Anti-MBLA (1/2)	40	45	40	30
Anti-Ia (1/1,000)‡‡	50	50	50	50
Anti-Ia (1/50)	70	70	70	70
Anti-Thy-1 + anti-Ia (1/1,000)	60	65	70	70
Anti-Thy-1 + anti-Ia (1/50)	>90	ND	ND	ND
Anti-MBLA + anti-Ia (1/1,000)	>95	>90	90	90
Anti-MBLA + anti-Thy-1	>95	100	>95	90
Anti-Ia <sup>k</sup> + anti-Ia <sup>a</sup>	ND	ND	ND	70

\* LNC, lymph node cells.

‡ I<sup>k</sup>, Thy-1.2.

§ I<sup>k</sup>, Thy-1.1.

|| I<sup>a</sup>, Thy-1.2.

\*\* Anti-Thy-1.2 (AKR/J anti-C3H/J) was used for all tests, except against A.AL LNC, where anti-Thy-1.1 (C3H/J anti-AKR/J) was used.

‡‡ Anti-I<sup>k</sup> (A.TH anti-A.TL) was used for all tests, except against A.TH LNC, where anti-Ia<sup>a</sup> (A.TL anti-A.TH) was used.

expressed on B cells or T cells or both. The 70% levels of cytotoxicity observed using lymph node targets, coupled with the 40% levels of B cells in lymph nodes (26), make restriction of Ia antigens to the B cell unlikely. Likewise, the 50–60% cytotoxicity of spleen cells makes a restriction to T cells also unlikely. Hammerling (15) has reported that the reactivity of an almost identical anti-Ia serum (B10.S × A.TH)- $\alpha$ -(B10.S × A.TL) is restricted to B lymphocytes. Sachs has reported a similar but much lower-titered anti-B-cell serum (13). Our own antiserum reacts not only with 70% of lymph node lymphocytes but with 40–50% of cortisone-resistant thymocytes. The cortisone-resistant thymocytes are greater than 95% sensitive to anti-Thy-1<sup>b</sup> sera. These data clearly demonstrate the reactivity of this anti-Ia serum with some population of T cells. Further, a T-cell (Thy-1-positive) tumor has also been found which is killed by the anti-Ia sera. Interestingly, when T cells from either spleen, lymph node, or cortisone-resistant thymus are purified by passage through nylon wool column, these T cells are no longer reactive with anti-Ia serum. Two experiments are particularly revealing. The finding that the nylon column selectively eliminates reactive cortisone resistant thymocytes may imply that the Ia-positive T cells are simply removed. However, the experiments in which recoveries are sufficiently high demonstrate not simply a depletion of Ia-positive cells, but a probable conversion of Ia-sensitive to Ia-insensitive cells. This capacity of cells to convert from anti-Ia-sensitive to Ia-insensitive may have important implications for attempts to determine the function of Ia antigens on lymphocyte surfaces by adding anti-Ia sera to block lymphocyte function in vitro in experiments in which nylon fiber purified cells are used as a source of T cells.



The Ia-reactivity of up to 60% of spleen cells from normal mice, the reactions of anti-Ia serum with nude spleen and lymph node cells, and the killing of PFC by anti-Ia sera, all unequivocally confirm the presence of Ia antigens on B cells, as found by Hammerling et al. (15) and Sachs and Cone (13).

The next important question is whether the T- and B-cell reactivities were caused by one or two discrete antibody populations. The biphasic titration curve is suggestive of two discrete antibody populations, each reacting with its own antigen. This suggestion is given further support by the single phase curve and high titer of cortisone-resistant thymocytes. Further support for this interpretation is generated by the following arguments. If there are present in the serum a high titered anti-T-cell antibody and a lower titered anti-B-cell antibody, then at low serum concentration (high dilution) the serum would appear specific for T cells. The prediction would then be for additive killing of anti-Ia with anti-MBLA, but not with anti-Thy-1<sup>b</sup>. When the anti-Ia serum was used at low concentration that is, of course, what was observed. It would also be predicted that at low concentrations, anti-Ia reactivity could be absorbed by T cells. As shown, the anti-Ia serum was absorbed both by cortisone-resistant and normal thymus, although less efficiently by normal thymus.

Another prediction of the two antibody-two antigen model would be that nude mice should be able to absorb the anti-B-cell activity without removing the anti-T-cell reaction. This was also observed. Sera absorbed in nudes retained activity both against cortisone-resistant thymus and normal cells. The observation that PFC are killed by high but not low concentrations of anti-Ia, even though the entire cell population shows significant cytotoxicity at low concentrations, is also consistent.

Two observations seem to argue against these interpretations. First, the titration curve with nude lymph node and spleen target cells is high titered. Since nude mice have few functional T cells, this suggests that the high-titered antibody is also directed at B cells. The second observation is that after absorption in vivo for 24 h, nude mice completely removed activity for normal BALB/c lymphocytes. In other anti-Ia absorptions, as well as with anti-*H-2D* or *H-2K*, antibodies not removed by 6 h are not usually absorbed by 24 h. This suggests that all of the antibodies can really be absorbed by B cells, although the antibody population most reactive with T cells is less efficiently absorbed. An alternative interpretation of these observations stems from recent observations that nude mice often have small but detectable numbers of T cells (26, 28) and increased numbers of peripheral T-cell precursors (29). If the antigen is expressed in nudes, either on the T cells present in low numbers, or on the T-cell precursors at low density, they might still be detectable by our sensitive cytotoxic test. These cells might react like mature T cells on direct test, even though their absorptive capacity might be much lower than normal cells. This would explain the slow in vivo absorption by nudes for T cells, but their rapid absorption of the B-cell antibody. Yet another explanation is that the affinity of the T-cell antibody is lower for B cells so that absorption of the T-cell antibody by nude cells is not efficient. This would also explain the slow absorption by nudes.

We have described above the tissue distribution of the Ia antigens and found

them to be present on both T and B cells. Further suggestive evidence has been presented indicating that there may be two discrete antibody populations, one specific for T cells and one for B cells. It is not yet known if any of the individual specificities thus far assigned in the Ia system (30, 31) are restricted to either T or B cells. The possible functions of these antigens remain to be determined. Are they the MLR determinants, or perhaps the T-B-cell recognition structures suggested by Katz et al. (18)? Are they the T-cell antigen receptors? Work is underway to attempt to answer these questions and to further define the function(s) of the Ia antigens.

### Summary

We have demonstrated in an anti-Ia serum the presence of specific antibodies reacting with T cells, as well as with B cells, using a highly sensitive dye exclusion test. This antiserum reacts with both spleen and lymph node in a characteristic biphasic titration curve killing up to 70% of these cells. It also reacts with cortisone-resistant thymocytes. The A.TH- $\alpha$ -A.TL serum can be absorbed with spleen, lymph node, cortisone-resistant thymus, or normal thymus cells. Further in vivo absorptions in BALB/c nude cannot remove all of the cytotoxic activity for normal BALB lymph node lymphocytes, while completely removing the activity for nude cells. A Thy-1 positive cell line derived from a C57Br leukemia is reactive with this anti-Ia serum.

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