

SECRETION OF Ia ANTIGENS BY A SUBPOPULATION OF  
T CELLS WHICH ARE Ly-1<sup>+</sup>, Ly-2<sup>-</sup>, AND Ia<sup>-\*</sup>

BY IAN F. C. MCKENZIE AND CHRISTOPHER R. PARISH

(From the Department of Medicine, Austin Hospital, Heidelberg, Victoria 3084, Australia, and the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia)

The Ia antigenic specificities have been detected predominantly on the surface of B cells and are coded for by genes within the *H-2* complex in the mouse (1, 2). The current interest in this antigenic system centers around its close genetic, and possibly functional relationship to the *Ir* genes, also found within the same *I* region of the *H-2* complex (1, 3). Accordingly, we have been investigating some of the properties and the role of Ia antigenic specificities in immune responses. We have recently published data showing that (a) Ia antigens, of low molecular weight, are found in serum (4) and in other tissue fluids and secretions (5); (b) while these specificities are found predominantly (but not exclusively) on the surface of B cells they are in fact produced by T cells (6). It is therefore of some importance to define the type of T cell which produces these specificities, especially in the light of recent data (7) which convincingly demonstrated that on the basis of Ly antigens, "helper" and "killer" T cells consist of different subpopulations of T lymphocytes.

In this paper we describe experiments that demonstrate that the subpopulation of splenocytes which secretes Ia antigens is nonadherent and has the surface phenotype of  $\theta^+$ , Ig<sup>-</sup>, Ia<sup>-</sup>, Ly-1<sup>+</sup>, Ly-2<sup>-</sup>.

### Materials and Methods

*Mice.* The mice used in the experiments are listed in the tables. CBA/H mice were raised at the Australian National University, Canberra, and all other mice at the Austin Hospital, Melbourne. These mice were originally obtained from The Jackson Laboratory, Bar Harbor, Maine. We are particularly grateful to Dr. Marianna Cherry of The Jackson Laboratory for the supply of C57BL/6-Ly-1<sup>a</sup> and B6.PL(75NS) strains which are congenic at the *Ly-1* and *Ly-2* loci, respectively, with C57BL/6.

*Antisera.* The characteristics of the antisera used are summarized in Table I. The anti-Ly and anti-Thy-1 sera were produced by immunizing recipients with six weekly injections of donor thymus cells, whereas in the case of the mouse anti-Ia serum, recipient mice received donor lymph node and spleen cells. One cell donor was used for every 5-10 recipients, and after the initial immunization regime, mice were bled and immunized at weekly intervals. These mouse antisera were tested by a cytotoxicity test using rabbit complement (4). The anti-Ly sera were considered to be specific for Ly-1.1 and for Ly-2.1 as (a) they were raised in donor-recipient combinations which are congenic for the *Ly-1* and *Ly-2* loci, respectively; (b) they reacted with the appropriate strains

\* Supported by research funds obtained from the National Cancer Institute, National Institutes of Health. Contract No. 1-CB-43925 (I. McKenzie) and grants obtained from the National Health and Medical Research Council and Tobacco Research Foundation, Australia.

TABLE I  
*Alloantisera Used in the Various Experiments*

Specificity	Donor	Recipient	Titer (% kill)*		
			Thymus	Spleen	Lymph node
Ly-1.1‡ (AS 379)	C57BL/6-Ly-1 <sup>a</sup>	(C57BL/6 × A)F <sub>1</sub>	>1/512 (100%)	1/256 (40%)	1/512 (70%)
Ly-2.1‡ (AS 457)	B6.PL(75NS)	(B10.AKM × 129)F <sub>1</sub>	>1/512 (85%)	1/32 (30%)	1/64 (50%)
Ia-1,2,3,7‡ (AS 383)	A.TL	A.TH	0	1/2,500 (65%)	1/2,000 (30%)
Thy-1.2	C3H	AKR	>1/1,000 (100%)	1/16 (30%)	1/32 (65%)

\* % kill: the maximum percent lysis of cells in the particular tissue using a cytotoxic test and rabbit complement.

‡ Raised in strains congenic at the various loci and considered therefore to be functionally specific.

(8) in accordance with their assigned specificities; and (c) autoantibodies were removed by extensively absorbing both sera with C57BL/6 thymus cells.

#### *Fractionation of Spleen Cell Populations*

**DEPLETION AND ENRICHMENT OF Ig<sup>+</sup> CELLS.** Cells bearing surface Ig were removed from a suspension of CBA/H spleen cells by forming Ig rosettes and separating the rosetting and nonrosetting cells by centrifugation on Isopaque/Ficoll (9). Briefly, sheep red cells (SRC) were coated, via CrCl<sub>3</sub>, with sheep anti-rabbit IgG and were mixed with CBA/H spleen cells which had been reacted with rabbit anti-mouse Ig. The mixture of SRC and spleen cells was centrifuged at 400 g, the cells suspended in their own supernate, and the rosette-forming cells subsequently sedimented using Isopaque/Ficoll as previously described (9). The Ig<sup>-</sup> cells were harvested from the Isopaque/Ficoll medium interface, and Ig<sup>+</sup> cells were recovered from the red cell pellet.

**DEPLETION OF CELLS BEARING Thy-1.2, Ly-1.1, Ly-2.1, OR Ia SURFACE ANTIGENS.** Spleen cells were treated with the different antisera for 30 min at 37°C, 10 ml of medium added, the cells sedimented, complement added, and a further 30-min period of incubation at 37°C performed, after which the cells were washed twice in medium (10% fetal calf serum (FCS) in phosphate-buffered saline) and their viability assessed. The amount of antiserum used was always adequate to lyse all cells of that particular specificity, the amounts being calculated, by extrapolation, from the cytotoxicity titer curves. To check this, retreatment of the cells with additional antiserum and complement was performed, and this did not lead to additional cell lysis. The antiserum treatments were as follows: (a) Anti-Thy-1.2:2 × 10<sup>8</sup> spleen cells in 1.6 ml were mixed with 0.4 ml of anti-Thy-1.2 (i.e., final dilution 1/5), incubated, and after washing 2 ml of guinea pig complement (GPC) was added at a dilution of 1/3. This complement was absorbed with agarose immediately before use. (b) Anti-Ly-1.1, anti-Ly-2.1, and anti-Ia-1, 2, 3, 7: cells were suspended in 1.0-ml aliquots containing 1.5 × 10<sup>8</sup> cells and exposed to 1.0 ml of the different antisera at 1/2 dilution. After incubation and washing, they were then treated with 1.3 ml of rabbit complement (RC) (dilution 1/3). After these treatments, the percentage of cells lysed was anti-Thy-1.2, 34%; anti-Ly-1.1, 42%; anti-Ly-2.1, 21%; anti-Ia, 58%; RC, 12%; and GPC, 8%. After treatment with antiserum, dead cells were removed by centrifugation on Isopaque/Ficoll (10).

**DEPLETION OF MACROPHAGES.** Macrophages were removed from the CBA/H spleen cell populations by first incubating the cells with carbonyl iron and then removing the phagocytic cells with a magnet as previously described (11).

**Tissue Culture Conditions.** The conditions of tissue culture have been previously described in detail (6). Briefly, the various spleen cell populations from CBA/H mice were cultured in Petri dishes in 10 ml of F15 medium (Grand Island Biological Co., Grand Island, New York) lacking FCS for 5 h at 37°C in 10% CO<sub>2</sub>, 7% O<sub>2</sub>. After incubation, the supernates were collected, dialyzed against distilled water using acetylated dialysis tubing in order to retain the low molecular weight material, and then freeze dried. The samples were reconstituted in 0.5 ml of distilled water before testing. In some experiments either sodium azide (0.1%) was added to the culture fluids or the spleen cells were preincubated with 10<sup>-5</sup> M Pactamycin for 60 min at 37°C before being washed and cultured as above. The Pactamycin was kindly supplied by Dr. B. Loughman, Upjohn Company, Kalamazoo, Mich.

TABLE II  
*Secretion of Ia Antigens by Various Subpopulations of Spleen Cells  
 In Vitro*

Spleen cell preparation cultured	Cell no.†	Inhibition of anti-Ia sera*	
		Mouse anti-Ia	Rabbit anti-Ia
Unfractionated	$2.0 \times 10^6$	160	640
Macrophage depleted	$1.8 \times 10^6$	160	640
Ig <sup>+</sup> fraction	$1.2 \times 10^6$	0	40
Ig <sup>-</sup> fraction	$0.8 \times 10^6$	160	400
Anti-Thy-1.2 treated	$1.3 \times 10^6$	20	40
GPC control	$1.9 \times 10^6$	80	320
Anti-Ly-1.1 treated	$1.2 \times 10^6$	0	0
Anti-Ly-2.1 treated	$1.6 \times 10^6$	80	320
Anti-Ia <sup>k</sup> treated	$0.9 \times 10^6$	80	320
RC control	$1.8 \times 10^6$	80	320
Sodium azide treated	$2.0 \times 10^6$	20	40
Pactamycin treated	$2.0 \times 10^6$	20	80

\* Results expressed as reciprocal of dilution which gave 50% inhibition of cytotoxicity (mouse anti-Ia) or rosetting (rabbit anti-Ia).

† Cell numbers cultured after treatment of  $2.0 \times 10^6$  spleen cells by the various procedures.

*Measurement of Ia Content in Tissue Culture Supernates.* The Ia content of the tissue culture supernates was measured by two inhibition techniques we have described in detail in our earlier papers (4, 5). The first assay measured the ability of serial dilutions of the sample to neutralize the cytotoxicity of the A.TH anti-A.TL serum. The second assay assessed the capacity of samples to inhibit the binding of rabbit anti-Ia antibodies to lymphocytes, the rabbit antibodies bound being detected by a rosetting procedure.

## Results

*Nature of the Cell-Secreting Ia Antigens.* The experimental approach was to measure the ability of subpopulations of spleen cells to secrete Ia antigens in vitro. The results are presented in Table II and confirm our earlier in vitro and in vivo findings that T cells, and not B cells, secrete Ia antigens (6). Thus, unfractionated spleen cells or those depleted of macrophages or Ig<sup>+</sup> cells secreted similar amounts of Ia antigen (both cytotoxicity and rosetting assays for Ia gave comparable results). However, when T cells were removed (anti-Thy-1.2 treatment) or when Ig<sup>+</sup> cells were cultured, virtually no Ia material was detected, i.e., the secretory cell is Thy-1.2<sup>+</sup>, Ig<sup>-</sup>, and is not a macrophage. Furthermore, this secretory T cell, although actively secreting Ia antigenic material, does not carry Ia on its cell surface, at least not in sufficient amounts to be detected with anti-Ia<sup>k</sup> serum and complement, as treatment of spleen cells with anti-Ia antiserum and RC had no greater effect on the secretion of Ia antigens than complement alone. When the spleen cells were treated with the different Ly antisera and complement the results were clearly different. Anti-Ly-1.1 treatment completely eliminated Ia antigen secretion, whereas anti-Ly-2.1 treatment was virtually without effect. Thus, the T-cell-secreting Ia is Ly-1.1<sup>+</sup>, Ly-2.1<sup>-</sup>, and carries little or no Ia antigen on its surface. Furthermore, for Ia secretion to occur an active cellular metabolism is required as both sodium azide and Pactamycin inhibited Ia secretion (Table II).

Finally, it should be emphasized that the anti-Ly and anti-Ia sera used in these experiments have been previously shown to eliminate the correct func-

tional subpopulations of T lymphocytes (12). For example, the anti-Ly-1.1 serum and complement kills helper and DTH T cells, whereas the anti-Ly-2.1 reagent removes cytotoxic T cells and T cells which suppress antibody responses (12).

### Discussion

The data presented in this paper demonstrate that the cell type which secretes Ia antigens in vitro is a nonadherent T lymphocyte which is Ly-1<sup>+</sup>, Ly-2<sup>-</sup>, and Ia<sup>-</sup>. Furthermore, the release of Ia antigens by this subpopulation of T cells is a metabolically active process as secretion is inhibited by sodium azide, an uncoupler of oxidative phosphorylation, and by Pactamycin, an inhibitor of protein synthesis (13).

Recently it has been reported that helper T cells are Ly 1<sup>+</sup>2<sup>-</sup> (7, 12), whereas cytotoxic T cells (7, 12) and T cells which suppress antibody responses are Ly 1<sup>-</sup>2<sup>+</sup> (12, 14). Thus on the basis of their cell surface phenotype, the cells that secrete Ia antigens cannot be suppressor or cytotoxic T cells and may well be helper T cells. Consistent with the helper T-cell origin of the Ia antigens is the observation that antigen-activated T cells release both nonspecific (15) and antigen-specific (16) helper factors which carry Ia antigens. We cannot be completely certain, however, that helper cells are the secretors of Ia antigen, as it appears that the T cells which mediate delayed-type hypersensitivity reactions are also Ly 1<sup>+</sup>2<sup>-</sup> and Ia<sup>-</sup> (12).

The Ia antigen which is secreted by T lymphocytes appears to be predominantly of a low molecular weight both in vivo (4, 5) and in vitro (Jackson, Parish, and McKenzie, in preparation) and is oligosaccharide in nature (5). Inasmuch as the T-cell-replacing factors which carry Ia antigens are glycoproteins of a much higher molecular weight (15, 16), it seems likely that the Ia oligosaccharides may have arisen by proteolytic cleavage of these glycoprotein precursors.

It is of interest that the T cells which secrete Ia antigens are not eliminated by treatment with anti-Ia serum and complement. The simplest interpretation of this observation is that the Ia-secreting cells have little or no Ia antigen on their surface. Such an interpretation is reminiscent of the old finding that antibody-forming cells have a very low density of antibody on their surface (17, 18).

### Summary

It was found that Ia antigens are rapidly secreted by a subpopulation of splenic T lymphocytes which are nonadherent and which express the surface phenotype Ly-1<sup>+</sup>, Ly-2<sup>-</sup>, and Ia<sup>-</sup>. Secretion of the Ia antigens was a metabolically active process which was inhibited by sodium azide and by Pactamycin, an inhibitor of protein synthesis.

*Received for publication 22 June 1976.*

### References

1. Shreffler, D. C., and C. S. David. 1975. The *H-2* major histocompatibility complex and the *I* immune response region: genetic variation, function, and organization. *Adv. Immunol.* 20:125.

2. David, C. S. 1976. Serological and genetic aspects of murine Ia antigens. *Transplant. Rev.* 30:299.
3. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.)*. 175:273.
4. Parish, C. R., A. B. Chilcott, and I. F. C. McKenzie. 1976. Low molecular weight Ia antigens in normal mouse serum. I. Detection and production of a xenogeneic antiserum. *Immunogenetics*. 3:113.
5. Parish, C. R., D. C. Jackson, and I. F. C. McKenzie. 1976. Low molecular weight Ia antigens in normal mouse serum. III. Isolation and partial chemical characterization. *Immunogenetics*. In press.
6. Parish, C. R., A. B. Chilcott, and I. F. C. McKenzie. 1976. Low molecular weight Ia antigens in normal mouse serum. II. Demonstration of their T cell origin. *Immunogenetics*. 3:129.
7. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly<sup>+</sup> cells in the generation of killer activity. *J. Exp. Med.* 141:1390.
8. Klein, J. 1973. List of congenic lines of mice. I. Lines with differences at alloantigen loci. *Transplantation (Baltimore)*. 15:137.
9. Parish, C. R., S. M. Kirov, N. Bowern, and R. V. Blanden. 1974. A one step procedure for separating mouse T and B lymphocytes. *Eur. J. Immunol.* 4:808.
10. Davidson, W. F., and C. R. Parish. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods*. 7:291.
11. Parish, C. R., and J. A. Hayward. 1974. The lymphocyte surface. II. Separation of Fc receptor, C'3 receptor and surface immunoglobulin-bearing lymphocytes. *Proc. R. Soc. Lond. B. Biol. Sci.* 187:65.
12. Vadas, M. A., J. F. A. P. Miller, I. F. C. McKenzie, S. E. Chism, F-W. Shen, E. A. Boyse, J. R. Gamble, and A. M. Whitelaw. 1976. Ly and Ia antigen phenotypes of T cells involved in delayed-type hypersensitivity and in suppression. *J. Exp. Med.* 144:10.
13. Cohen, L. B., I. H. Goldberg, and A. E. Herner. 1969. Inhibition by pactamycin of the initiation of protein synthesis—effect on the 30S ribosomal subunit. *Biochemistry*. 8:1327.
14. Feldman, M., P. C. L. Beverley, M. Dunkley, and S. Kontiainen. 1975. Different Ly antigen phenotypes of *in vitro* induced helper and suppressor cells. *Nature (Lond.)*. 258:614.
15. Armerding, D., D. H. Sachs, and D. H. Katz. 1974. Activation of T and B lymphocytes *in vitro*. III. Presence of Ia determinants on allogeneic effect factor. *J. Exp. Med.* 140:1717.
16. Taussig, M. J., A. J. Munro, R. Campbell, C. S. David, and N. A. Staines. 1975. Antigen-specific T-cell factor in cell cooperation. Mapping within I region of the H-2 complex and ability to cooperate across allogeneic barriers. *J. Exp. Med.* 142:694.
17. Nossal, G. J. V., and H. Lewis. 1972. Variation in accessible cell surface immunoglobulin among antibody-forming cells. *J. Exp. Med.* 135:1416.
18. Perkins, W. D., M. J. Karnovsky, and E. R. Unanue. 1972. An ultrastructural study of lymphocytes with surface-bound immunoglobulin. *J. Exp. Med.* 135:267.