References and Notes

1. H. Beauregard, *Les Insectes Vésicantes* (Felix Alcan, Paris, 1890).
2. E. Kaiser and H. Michl, *Die Biochemie der Tierischen Gifte* (Deuticke, Vienna, 1958); L. Nicholls and D. Teare, *Br. Med. J.* **2**, 1384 (1954).
3. G. D. H. Carpenter, *Trans. R. Entomol. Soc. Lond.* **1921**, 1 (1921); H. Donisthorpe, *Entomol. Rec.* **16**, 150 (1904); F. M. Jones, *Trans. R. Entomol. Soc. Lond.* **80**, 345 (1932); G. A. K. Marshall, *ibid.* **1902**, 287 (1902); R. B. Selander and J. M. Mathieu, *Univ. Ill. Biol. Monogr. 41* (1969); R. J. Tafanelli and J. C. Bass, *Southwest. Nat.* **13**, 51 (1968).
4. L. Cuénot, *Bull. Soc. Zool. Fr.* **15**, 126 (1890).
5. Cleansing activities are the characteristic response of ants to defensive substances of their prey: T. Eisner, J. Meinwald, A. Monro, R. Ghent, *J. Insect Physiol.* **6**, 272 (1961).
6. The tubes were calibrated on the basis of their measured (ocular micrometer) inner diameters. Consumption rates were calculated from measurements of meniscus movement (corrected for evaporative loss of fluid).
7. Statistically the 20 tubes in the controls signified choice between 0.1*M* glucose and 0*M* cantharidin. Drinking rates were measured only from those 10 tubes that were randomly designated to correspond to 0*M* cantharidin.
8. The emulsion consisted of cholesterol, Tween 20, olive oil, and glass distilled water in the ratio of 1:3:3:20 by weight. Cantharidin was dissolved in the hot lipid phase before addition of water.
9. Blood samples were treated with concentrated HCl, and the hydrolysate was taken up in hot acetone, dried in a stream of nitrogen, and dissolved in chloroform for injection into the gas chromatograph. Two columns were used for each sample: 3 percent Silicone DC 560 (F60) on Chromosorb P 60/80 mesh, 6 feet by ⅛ inch (operated isothermally at 200°C), and 8 percent SE30 and 4 percent NPGS on Chromosorb W 08/100 mesh, 8 feet by ⅛ inch (programmed at 4°C per minute, 140° to 180°C). Operating conditions for both columns were identical: injector and flame ionization detector temperatures 250° and 275°C; air, hydrogen, and nitrogen flows 140, 14, and 28 ml/min, respectively. Correspondence of the designated peaks to cantharidin was unequivocally confirmed by gas chromatography–mass spectrometry (authentic cantharidin supplied by Inland Alkaloid Co.).
10. V. G. Dethier, *The Physiology of Insect Senses* (Methuen, London, 1963).
11. R. B. Selander, *Univ. Ill. Biol. Monogr. 28* (1960).
12. J. Meynier, *Arch. Med. Pharm. Mil.* **22**, 54 (1893).

13. We thank D. S. Robson for advice on statistical procedure, J. Hribar and J. Duxbury for mass spectrometric analyses, R. B. Selander for live Meloidae, F. G. Werner and W. R. Enns for identification of Meloidae, and W. L. Brown, Jr., for identification of ants. Study supported by NIH grant AI-02908 (T.E.) and NIH fellowship 5-FO1-GM40210 (J.E.C.). Paper No. 37 of the series *Defense Mechanisms of Arthropods.*

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B-Cell Alloantigens Determined by the H-2 Linked Ir Region Are Associated with Mixed Lymphocyte Culture Stimulation

Abstract. *Cell surface antigens, controlled by genes located in the Ir region of the murine major histocompatibility complex, are shown serologically to be expressed preferentially on bone marrow–derived lymphocytes. These antigens may play a major role as stimulators in mixed lymphocyte cultures.*

Recent developments in several different fields of immunology have focused attention on H-2, the murine major histocompatibility complex (MHC). Analysis of this region has led to the identification of four closely linked subregions in the linear order: H-2K, Ir, Ss-Slp, and H-2D. These are conventionally written from left to right with respect to the centromere of the ninth linkage group (*1*) (Fig. 1a). H-2K and H-2D genes code for serologically detectable antigens on cell membranes which seem to be important in graft rejection (*1*). To the right of H-2K lies the Ir region, which appears to control the ability of particular strains to generate an immune response to a large range of synthetic and naturally occurring antigens (*2*). Ir has been further subdivided into Ir-IgA and Ir-IgG loci which control the ability of mice to make antibody against myeloma proteins of these two classes (*3*). Ss governs the concentration of a serum protein of unknown function, and Slp, which as yet has not been genetically separated from Ss, controls production of an alloantigen of this protein expressed only in males (*4*).

Through the use of congenic resistant mouse strains, which are genetically identical except for their MHC, it has been shown that differences in the products of the MHC are necessary and sufficient to cause stimulation in mixed lymphocyte culture (MLC) (*5*). In this test, lymphocytes from mice of two different strains are placed into culture together. If the cells of one strain recognize cell surface antigens of the other as "foreign" they are stimulated to proliferate. By preventing proliferation of one population with mitomycin C, a "one-way MLC" can be obtained (*5*).

Several mouse strains have been developed in which the MHC contains a known crossover between H-2K and H-2D regions. Use of these recombinant strains in MLC testing has revealed that differences in the Ir region give the strongest MLC stimulation and that differences in the H-2K or H-2D regions with identity in the Ir region give weak stimulation (*5*). This has led to the hypothesis of multiple MLC loci of varying strength spread throughout the MHC (*6*). In examination of intra H-2 recombinants by MLC, an unusual result has been repeatedly observed with the congenic resistant strains B10.A(1R), B10.A(2R), and B10.A(4R) (abbreviated 1R, 2R, and 4R) (Fig. 1a). The strains 1R and 2R are identical with respect to known markers. In tests of cells from these three strains, 4R cells give a low but consistently significant response to 1R and 2R cells, but 1R and 2R cells do not respond above background when stimulated by 4R cells (*5*, *6*). The same result has also been seen when the 2R and 4R strains are examined for graft-versus-host reactivity by a Simonsen spleen weight gain assay (*7*): 4R cells produce significant graft-versus-host reactivity when injected into 2R mice, but no graft-versus-host reactivity is seen in the reverse combination.

In this report, we propose a genetic model to explain these "one-way" MLC and graft-versus-host reactivity results. The model, illustrated in Fig. 1b, invokes the existence of a distinct MLC locus located in Ir and of a deletion of this locus during the formation of the 4R recombinant MHC. If, during gametogenesis in the H-$2^{a/b}$ hybrid, an improper pairing of the MHC chromatids took place, then the crossover shown in solid line would not include either the H-2^a or the H-2^b MLC locus, resulting in a deletion. Such improper pairing may occur in regions containing a series of genes coding for similar functions (*8*). The Ir region has been thought to consist of a series of loci coding for membrane receptors (*2*), so it is conceivable that these loci would have regions of homology leading to improper pairing. Assuming a single crossover event, the postulated MLC locus must lie between Ir-1 and Ir-IgG, given the knowledge that 2R and 4R have the same Ir-1 but differ at Ir-IgG (*3*).

In order to test this model, congenic B10 anti-B10.A and B10.A anti-B10 antiserums were raised by the use of reciprocal skin grafts followed by weekly intraperitoneal injections of 20×10^6 lymphoid cells. These antiserums were absorbed with a number of 2R or 4R lymphoid cells sufficient to completely remove cytotoxic activity to the absorbing cell type (8×10^8 cells per milliliter of antiserum) and were

then tested against lymph node cells in a two-stage dye exclusion cytotoxicity test (*9*). The results, shown in Table 1, are consistent with the postulated genetic model. Antiserum 771 (B10 anti-B10.A) absorbed with 4R cells and tested on 2R cells was cytotoxic to 40 percent (average of several experiments was 32 ± 8 percent, S.E.) suggesting that a subpopulation of cells was being killed. When tested against B10.A cells, this absorbed serum was still capable of producing complete lysis with a titer of 1 : 16, as expected, since activity against the H-2D region ought not to be reduced by this absorption. This result also indicates that the incomplete lysis seen against 2R cells cannot be explained simply by anticomplementary effects produced by the absorption (*9*). This absorbed antiserum, according to our model, when tested against 2R cells, could theoretically detect products of the MLC, Ir-IgG, and Ss-Slp regions. Conversely, antiserum 771, absorbed with 2R and tested against 4R, should not have any residual activity, and it indeed did not (Table 1). Similarly, according to the model, antiserum 770 (B10.A anti-B10) absorbed with 2R and tested against 4R could theoretically detect products of the H-2^b Ir-IgG and Ss-Slp regions. However, as seen in Table 1, no activity could be detected. Again, tests against the initial B10 immunizing cells showed high activity, eliminating the possibility of anticomplementary effects. These cytotoxic data thus show "one-way" reactivity similar to that seen in MLC and graft-versus-host reactions of 2R and 4R and are consistent with the hypothesis that the same cell surface antigen (or antigens) which is detected by our absorbed B10 anti-B10.A antiserum is responsible for the "one-way" cellular reactions between these strains.

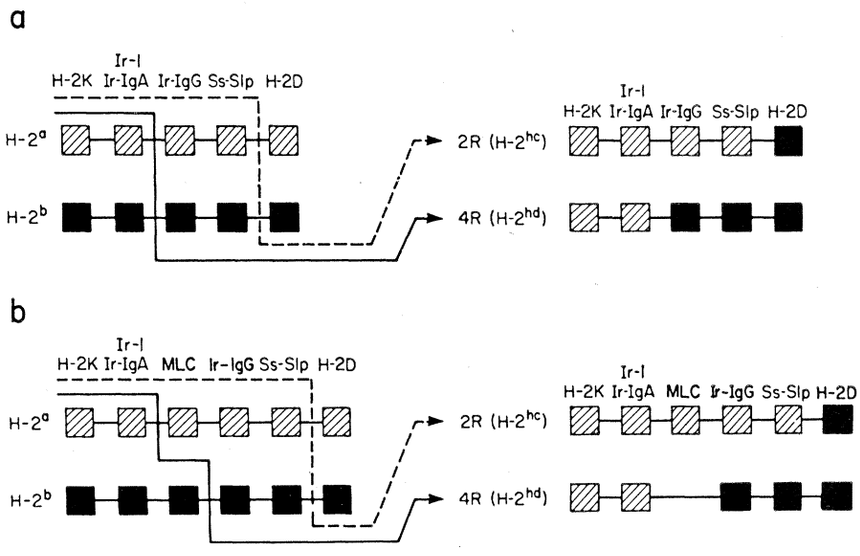

Fig. 1. (a) Conventional model for the derivation of the MHC regions of 2R and 4R recombinants. (b) Unequal crossover model for the derivation of the MHC regions of 2R and 4R recombinants. For schematic purposes, the recombinants are shown arising from an H-2^a (B10.A) × H-2^b (C57BL/10) mating. In actuality, they appeared during the matings leading to the formation of the B10.A congenic resistant strain.

Table 1. Cytotoxicity of absorbed alloantiserums against lymph node cells of indicated strains.

Absorbing cells	Target cells	Lysis (%)*		
		Complement control	Maximum	Specific (net)
		Serum: 771 (B10 anti B10.A)		
2R	2R	18	18	0
2R	4R	13	17	4
2R	B10.A	< 10	> 80	> 70
4R	2R	< 10	50	40
4R	4R	13	12	0
4R	B10.A	< 10	> 80	> 70
		Serum: 770 (B10.A anti B10)		
2R	2R	< 10	< 10	0
2R	4R	25	27	2
2R	B10	< 10	> 80	> 70
4R	2R	14	17	3
4R	4R	24	28	4
4R	B10	< 10	> 80	> 70

* Media controls all < 10 percent. Data are from a single representative experiment. Each number represents 100 cells counted in randomly selected fields.

Table 2. Cytotoxicity of B10 anti-B10.A alloantiserum absorbed with 8 × 10^8 4R cells per milliliter and tested against fractionated 2R cells.

Target cells*	Ig positive (%)	Lysis (%)		
		Complement control	Maximum	Specific (net)
2R Unseparated	49	16	40	24
2R Nonadherent ("T")	2	28	35	7
2R Adherent ("B")	83	16	85	69

* Unabsorbed antiserum killed more than 80 percent of all three populations with similar titers. Separations were kindly performed by Dr. B. S. Handwerger and Dr. R. H. Schwartz.

We separated 2R lymph node cells over a nylon wool column into populations enriched for T cells (nonadherent) and B cells (adherent) (*11*). Tests in the cytotoxicity assay with antiserum 771 absorbed with 4R (Table 2) showed that the level of killing correlated well with the percentage of cells which were Ig positive. This indicates, then, that the putative MLC antigen is expressed preferentially on B cells. This finding is compatible with current evidence that

The full titration curve of the cytotoxic reaction of 2R cells showed a plateau of killing at the 32 percent level from a dilution of 1 : 2 to a dilution of 1 : 16, indicating that the fraction of cells killed could not be increased by increasing the concentration of the relevant antibodies. Analogous patterns of cytotoxic activity have recently been reported from our laboratory for the reaction of a B10.A anti-B10.D2 alloantiserum tested against C57BL/10 lymph node cells (*10*). In this case, the cell surface antigen (tentatively called "β") responsible for the reactions was encoded by a gene also mapping to the left of Ir-IgG. The subpopulation of reactive cells was determined by a variety of criteria to be B cells. We therefore attempted similar classification of the subpopulation bearing this putative MLC antigen.

B cells (obtained from "nude" athymic mice) stimulate as well as normal cells in MLC (*12*). It is possible that the B cell antigen ("β") which we have described previously may also be responsible for stimulation in MLC. In fact, "β" could be an allele of the antigen described in this report. Its map location is consistent with this, as is the observation that all strains thus far tested express either "β" or this MLC-associated antigen, but not both. There may also be a variety of other MLC loci elsewhere in the MHC, as has been previously suggested (*6*).

Functionally, the finding of a B cell antigen mapping in Ir may be significant, since the precise mechanism of Ir gene expression has not yet been resolved. Independent evidence exists for defects in the T and in the B populations of nonresponder strains (*2, 13*). Thus it is possible that the Ir region codes for a series of membrane antigens, some expressed on T cells (*14*) and others on B cells (*10*), governing responsiveness for a broad series of antigens. On the other hand, the genetic mapping of such lymphoid differentiation antigens could be fortuitous, and a definitive association with Ir must await demonstration of functional relationships.

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References

1. H. O. McDevitt, B. D. Deak, D. C. Shreffler, J. Klein, J. H. Stimpfling, G. D. Snell, *J. Exp. Med.* **135**, 1259 (1972).
2. B. Benacerraf and H. O. McDevitt, *Science* **175**, 273 (1972).
3. R. Lieberman, W. E. Paul, W. Humphrey, Jr., J. H. Stimpfling, *J. Exp. Med.* **136**, 1231 (1972).
4. H. C. Passmore and D. C. Shreffler, *Biochem. Genet.* **5**, 201 (1971).
5. F. H. Bach, M. L. Bach, P. M. Sondel, G. Sundharadas, *Transplant. Rev.* **12**, 30 (1972).
6. F. H. Bach, M. B. Widmer, M. L. Bach, J. Klein, *J. Exp. Med.* **136**, 1430 (1972); T. Meo, J. Vives, V. Miggiano, D. Shreffler, *Transplant. Proc.* **5**, 377 (1973).
7. S. Livnat, J. Klein, F. H. Bach, *Nat. New Biol.* **243**, 42 (1973).
8. A. M. Srb and R. D. Owen, *General Genetics* (Freeman, San Francisco, 1952), pp. 199–200.
9. D. H. Sachs, H. J. Winn, P. S. Russell, *J. Immunol.* **107**, 481 (1971).
10. D. H. Sachs and J. L. Cone, *J. Exp. Med.* **138**, 1289 (1973).
11. M. H. Julius, E. Simpson, L. A. Herzenberg, *Eur. J. Immunol.* **3**, 645 (1973); B. S. Handwerger and R. H. Schwartz, manuscript in preparation.
12. C. Cheers and J. Sprent, *Transplantation* **15**, 336 (1973).
13. E. Mozes and G. M. Shearer, *Curr. Top. Microbiol. Immunol.* **59**, 167 (1972).
14. V. Hauptfeld, D. Klein, J. Klein, *Science* **181**, 157 (1973).

3 October 1973

Osmotic Gradients across Snail Epidermis: Evidence for a Water Barrier

Abstract. *Cryoscopic analysis of frozen sections provided indirect evidence for the presence of a waterproof layer limiting evaporation from living epithelial cells in dormant land snails.*

Although large water concentration differences commonly exist across the skins of terrestrial animals (*1*), living cells in these layers are usually protected from desiccation by waterproof stratified epithelia in vertebrates and by cuticles in the invertebrate phyla. However, the single-layered molluscan epidermis secretes no cuticle, bearing instead a zone of microvilli along its free apical surface (*2*). Those areas of a land snail's skin not covered by the shell are normally protected by the sustained secretion of watery mucus. When snails withdraw into the shell, a limited area of mantle tissue remains exposed to the air at the aperture. During dormancy, which sometimes lasts for periods exceeding a year (*3*), cutaneous secretion in this area almost completely ceases, and evaporative water loss is drastically decreased (*4–6*). Surprisingly, the levels to which evaporation is reduced are similar to those found in some insects (*7*). In a study involving surface temperature measurements (*6, 8*), evaporation appeared to be retarded by a waterproof barrier, not immediately at the mantle surface, but located beneath a superficial compartment in equilibrium with the ambient air and freely exchanging water with it. This superficial compartment behaves as if it were composed of mucus, which has been shown in earlier studies (*5, 6*) to be hygroscopic but not particularly impermeable to water, even when air-dried. The absence of an impermeable layer on the surface suggests that the barrier might be located within or beneath the epithelial cells. If this were the case, structures outside the barrier would be subjected to atmospheric dehydration.

As a means of locating the impermeable barrier by the depth to which dehydration extends into the tissue, I recorded the distribution of melting points in frozen transverse sections of mantle tissue. Dormant *Otala lactea* (Müll.) were kept several weeks without disturbance (*9*) before quick freezing in isopentane cooled to −140°C with liquid nitrogen. Sections 4 μm thick were placed on a perforated, temperature-controlled silver block and mounted in kerosene under a glass cover slip. After sufficient time for temperature equilibration to abolish thermal gradients, the frozen specimens were photographed by conventional transmitted light microscopy at regular temperature intervals from −10°C to final melting.

A superficial zone, similar to that found in frog skin (*10*), remained unfrozen even at −30°C, which suggests very high solute concentrations (*11*). This layer probably consists of dehydrated mucus since it is exterior to the epithelial cells and swells slightly when a significant proportion of the section

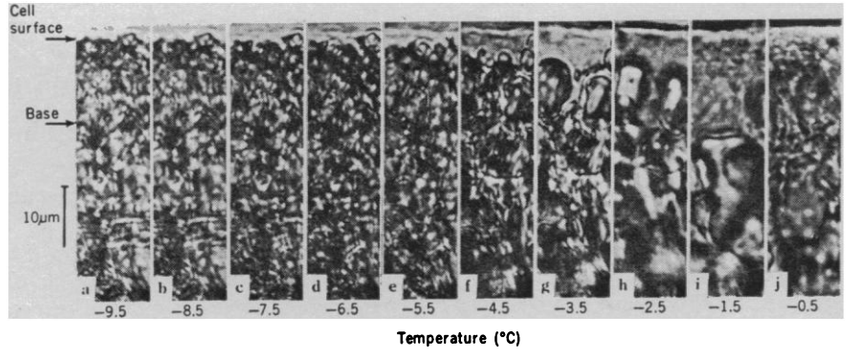

Fig. 1. Frozen section of mantle epithelium photographed at progressive stages of melting. Ice crystals appear as a semiopaque, granular mass beneath the superficial, transparent unfrozen zone. The ice front retreats gradually with temperature in (a) to (f) and more rapidly in (g) to (i). The ice in (j) is completely melted. Increase in individual crystal size just before melting is seen particularly in (f) to (i). The upper and lower limits of the epithelial cells are indicated by the arrows. Their position, determined by the slight change in ice texture at the base, was checked later with stained sections from the same tissue.