ANTIGEN BINDING AND CAPPING BY LYMPHOCYTES OF GENETIC NONRESPONDER MICE*

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Single dominant genes intimately linked to the major histocompatibility locus, H-linked specific Ir genes, control the immune response to many antigens with limited structural diversity (1). The response of mice to the linear random copolymer $(\text{L-glu}^{60}\text{L-ala}^{30}\text{L-tyr}^{10})_n$ (GAT) is governed by a single dominant gene lying within the *H-2* region of the ninth linkage group. Mice possessing the alleles $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$, or $H\text{-}2^k$ uniformly respond to immunization with GAT by making antibody in high titer, while mice with the $H\text{-}2^p$ or $H\text{-}2^s$ alleles make no antibody detectable by a sensitive Farr assay in response to the same immunization procedure (2–4). Considerable evidence has been presented that H-linked Ir genes are necessarily expressed in thymus-derived cells and in some way control their ability to respond to antigens (1).

All immune responses which are attributable to the activity of thymus-derived cells (delayed hypersensitivity, in vitro manifestations of cellular immunity, carrier function, etc.) are found to be totally dependent on the possession of the relevant Ir gene. Furthermore, in several systems the ability to form antibodies against certain determinants of the antigen is not lacking in nonresponder animals and can be demonstrated by immunizing with the antigen complexed with an immunogenic carrier. These findings suggest the presence in nonresponder animals of normal B lymphocytes with specific immunologic receptors for the antigen. To investigate this issue directly, we have studied by radioautography the binding of highly labeled GAT by spleen cells of genetic responder and nonresponder mice.

Materials and Methods

Mice.—Mice of strains A/HeHa ($H\text{-}2^a$) and C3H_f/HeHa ($H\text{-}2^k$) (responders to GAT) were obtained from West Seneca Laboratories, Buffalo, N. Y. Mice of strains P/J ($H\text{-}2^p$) and SJL/J ($H\text{-}2^s$) (nonresponders to GAT) were obtained from the Jackson Laboratory, Bar Harbor, Maine. Male mice between 6 and 15 wk of age were used.

Antigens and Immunization.—GAT (mol wt ~45,000) was synthesized to order by Pilot Chemicals, Inc., Watertown, Mass. Mice were injected intraperitoneally with GAT with magnesium-aluminum hydroxide and pertussis as previously described (3) 4–7 wk and 1 wk

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before the experiments. Keyhole limpet hemocyanin (KLH) was obtained from giant keyhole limpets (*Megathura crenulata*) and purified by ultracentrifugation. To elicit an antibody response in nonresponder mice, GAT was complexed with methylated bovine serum albumin (MBSA) according to a technique developed by Dr. Paul H. Maurer. Details of the immunization are given in Table II.

Radioiodination.—GAT and KLH were conjugated with ^{125}I (carrier-free Na^{125}I, New England Nuclear Corp., Boston, Mass.) by the chloramine-T method (5) in microvolumes and the product separated from unconjugated ^{125}I by gel filtration on 0.5 × 25 cm columns of Sephadex G-25F (Pharmacia Fine Chemicals, Piscataway, N. J.). For radioautography specific activity of the antigens ranged from 25 to 100 μCi/μg while in the antigen-binding assay specific activity of GAT ranged from 1 to 4 μCi/μg.

Antigen-Binding Assay.—Antigen-binding assays were performed on samples of plasma diluted 1:5 in saline (3).

Preparation of Cell Suspensions.—Spleen cells were havrested by conventional methods using Eagle's minimal essential medium without L-glutamine fortified with 10% fetal calf or bovine serum (Microbiological Associates, Bethesda, Md.) (tissue culture medium, TCM). Cells were incubated with 2 μg radiolabeled antigen per 10^7 cells in 0.5 ml TCM on ice and washed five to eight times with TCM to remove nonspecifically bound antigen before proceeding. They were finally suspended in 0.05–0.1 ml TCM and smears of 0.01 ml were made on slides previously coated with 0.5% gelatin. The slides were air-dried and fixed in absolute methanol.

In the initial experiment the cell suspension from two spleens in 5 ml was layered on a 3 ml Ficoll-Hypaque gradient (12 parts 9% Ficoll [Pharmacia Fine Chemicals] + 5 parts 34% Hypaque [Winthrop Laboratories, New York]), centrifuged at 275 *g* for 25 min at room temperature, and the gradient and interface aspirated, washed twice with 10 ml TCM, counted, and resuspended before proceeding with the incubation with radioactive antigen. Because of the tendency of the highly ionized GAT to adhere nonspecifically to dead cells, protein in the medium, and debris, it was found preferable to put the cell suspension through the Ficoll-Hypaque gradient after reacting it with radiolabeled antigen. This procedure was followed except in the experiment comparing the effects of incubating the labeled cells in the warm and in the cold.

Radioautography.—Kodak Nuclear Tracking Emulsion NTB2 (Eastman Kodak Company, Rochester, N. Y.) and Ilford Nuclear Research Emulsion L4 (Ilford, Ltd., Ilford, Essex, England) were used according to conventional techniques. Slides were incubated at 4°C for 5–20 days before developing.

RESULTS

Frequency of GAT-Binding Cells.—The vast majority of cells binding GAT appeared to be typical small lymphocytes in both responders and nonresponders. A few cells recognizable as macrophages bound small amounts of antigen; these were excluded from the counts. The frequency of GAT-binding cells in the spleens of mice of two responder (A and C3H) and two nonresponder (P and SJL) strains was determined in two experiments (Table I). There was no significant difference in the number of antigen-binding cells between responders and nonresponders; the frequencies range between 2.4 and 10 binders per 10^4 cells in both groups of animals and fall within the rather wide range reported in other systems (6–9). However, responders differ from nonresponders in their response to injection with antigen: while the frequency of binders increases modestly (2.4–3.7-fold) after immunization in responders, in nonresponders it scarcely changes. The small increase with immunization in re-

sponders is again within the rather wide range found in other systems (6). The number of grains per cell, although not systematically evaluated, appeared not to differ between responders and nonresponders.

Presence in Nonresponders of Cells Capable of Forming Antibody.—As has been demonstrated in other systems (10), nonresponding mice immunized with

TABLE I

*Proportions of Spleen Cells Binding GAT-^{125}I × 10^{4}**

	Not injected with antigen	Injected with antigen‡
Responder strains		
C3H	4.1	14.7
A	3.1	7.3
Nonresponder strains		
P	2.4	3.8
SJL	10	8.0

* 20,000–53,000 cells were examined in each case.

‡ Animals injected intraperitoneally with 100 μg GAT with magnesium-aluminum hydroxide and pertussis 4–7 wk and 1 wk before the experiment.

TABLE II

Immune Response to GAT Coupled to MBSA

Strain	Immunization	% binding of GAT*	
		Day 14 (primary)	Day 22 (secondary)
C3H	GAT-MBSA 10 μg	28.0	54.1
SJL	GAT-MBSA 10 μg	20.2	52.4
	GAT-MBSA 100 μg	25.2	63.4
C3H	GAT 100 μg	80.2	77.5
SJL	GAT 100 μg	<1	2.9

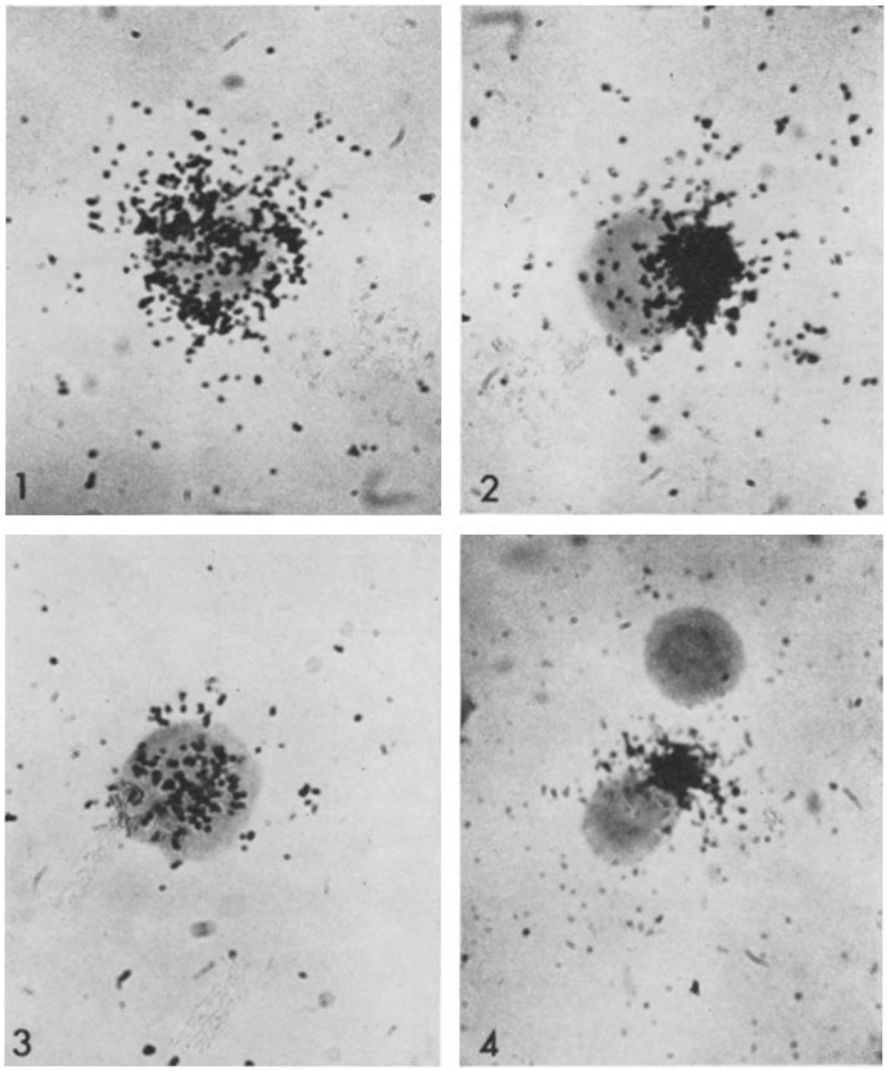
Mice received GAT-MBSA in complete Freund's adjuvant (Difco Labs, Detroit, Mich.) and GAT in alum-pertussis vaccine (3) twice at 2-wk intervals.

* Arithmetic means of values from four or five animals per experimental group. Values represent the per cent binding of 25 ng of GAT-^{125}I by 25 μl of 1:5 dilution of plasma.

GAT complexed to MBSA made as much antibody as the responding mice (Table II and similar results of Dr. Paul H. Maurer).

Pattern of Binding of Radiolabeled Antigen to Cells.—In the initial experiments, when the cells were kept on ice from the time of incubation with antigen to the time of smearing, the silver grains in the radioautographs were distributed in a patchy fashion all over the labeled cells. Because this technique left a certain amount of debris in the suspension to which the labeled GAT tended to adhere, we adopted the practice of sedimenting the cells on a Ficoll-Hypaque gradient just before smearing them. As the constituents of the gradient became insoluble below 15°C, this procedure was carried out at room temperature and the cells were thus warmed for 25 min. Under these circumstances we found that the silver grains of the radioautograph tended to be bound in a clump at

one pole of the cell in responders and nonresponders alike, whether or not injected with antigen, forming a typical cap.



FIGS. 1–4. Radioautographs of spleen cells from A/HeHa mice with radioiodinated antigens. (*1*) Cell incubated with KLH-^{125}I and kept at 4°C. (*2*) Cell incubated with KLH-^{125}I and warmed to 37°C for 20 min. (*3*) Cell incubated with GAT-^{125}I and kept at 4°C. (*4*) Cell incubated with GAT-^{125}I and warmed to 37°C for 20 min. Magnification 1000.

We further investigated this apparent movement of the cell-bound antigen in spleen cell suspensions from A strain mice using either radiolabeled KLH or GAT. After incubation with radiolabeled antigen for 30 min in the cold, one

preparation which had been treated with each antigen was placed in a 37°C water bath for 20 min while another was kept on ice for the same time, after which all preparations were quickly washed once in the cold and smeared. The majority of spleen cells (76–93%) kept at 4°C exhibited grains over the entire surface. Warming the cells to 37°C led to a change in the pattern of distribution in that most cells (73–86%) now exhibited grains in one area of the cell encompassing usually about ⅓ to ½ its surface (Figs. 1–4). The number of grains in cells at 4° and at 37°C was approximately the same (and highly variable from cell to cell), indicating that the change in pattern was associated with a redistribution of the bound antigen rather than with a loss of part of it. Peritoneal macrophages were also studied. At 4°C the antigen was present over the entire cell. After incubation at 37°C it was observed in random areas in the cell. We could not discriminate between antigen bound to the macrophage surface and antigen within vesicles.

DISCUSSION

No significant differences could be detected in the ability of lymphocytes from mice possessing or lacking the H-linked Ir gene controlling the response to GAT to bind this antigen. Similar numbers of GAT-binding cells were detected in both responder and nonresponder mice. This finding explains the ability of nonresponder mice to synthesize anti-GAT antibody when immunized with GAT complexed with an immunogenic carrier and establishes the existence of GAT-specific precursors of antibody-secreting cells in these animals. The inability of *H-2*^{p} and *H-2*^{s} mice to respond to GAT must therefore be attributed to a genetic defect in the thymus-derived cell population. As expected, the absence of response in mice lacking the gene was associated with a failure to detect an increase in GAT-binding cells after immunization which is observed in genetic responders.

In the course of this study we have observed that antigen concentrates on the surface of antigen-binding cells in the form of a cap (Figs. 1–4). This phenomenon was observed previously and studied in detail (11)[1] when B lymphocytes were reacted with anti-immunoglobulin at 37°C and results from the cross-linking of antigen receptors on the cell membrane by the multivalent ligand. It is very probable that the presence of several repeating determinants on the antigen molecule is required for capping to occur with antigen. Both GAT and polymeric KLH would be expected to possess repeating determinants. As in the case of capping with anti-immunoglobulin, capping by specific antigen was seen to be highly dependent on temperature.

The ability of lymphocytes that bind GAT to display cap formation irrespective of the responder status of the animals indicates that the genetic defect in nonresponder animals cannot be attributed to an absence of cap formation by

[1] Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* In press.

antigen-binding cells, a phenomenon which is believed to precede the interiorization of the antigen and the stimulation of the cell.

SUMMARY

Radioautographic study of the binding of GAT-^{125}I to spleen cells of genetic responder and nonresponder mice demonstrates that among mice not injected with antigen all strains have approximately the same number of antigen-binding cells; after injection with antigen the number of antigen-binding cells increases in responders but not in nonresponders. Nonresponders are shown to make antibody after injection with GAT complexed with an immunogenic carrier, demonstrating the presence of potentially functional B cells in responders and nonresponders alike. When incubated in the warm, antigen-binding cells of both responders and nonresponders concentrate antigen at one pole of the cell, forming caps.

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