CELLS INVOLVED IN THE IMMUNE RESPONSE

XVI. The Response of Immune Rabbit Cells to Phytohemagglutinin, Antigen, and Goat Anti-Rabbit Immunoglobulin Antiserum*

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In a preceding article, evidence was presented for the existence of two distinct populations of lymphocytes in the normal adult rabbit—one capable of responding to stimulation with phytohemagglutinin (PHA)[1] and the other to goat antiserum to rabbit immunoglobulins (1). The question which these results pose is whether one or both of these functionally different populations of cells are capable of reacting to common protein antigens. This invesigation was designed to provide the answer to this question. The results clearly imply that only the lymphocytes which react to anti-immunoglobulin antiserum react to stimulation with specific antigen, with both of these reagents probably interacting with the same receptor site on the lymphocyte surface.

Materials and Methods

Adult, New Zealand white rabbits, varying in age from 4 to 6 months, were used in this study.

The phytohemagglutinin used was phytohemagglutinin M (PHA), obtained from Difco Laboratories, Inc., Detroit, Mich. The contents of a vial were dissolved in 5 ml of sterile Medium 199 (Med-199) (Microbiological Associates, Inc., Bethesda, Md.) and this solution, referred to as undiluted PHA, was diluted 5- and 10-fold with sterile Med-199.

Normal goat gamma globulin (NGGG) was obtained from Pentex, Inc., Kankakee, Ill.

The anti-rabbit immunoglobulin antiserum (GARIG) was obtained by immunizing adult goats. The preparation and characteristics of the antiserum have been described in detail

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[1] *Abbreviations used in this paper:* GARIG, goat anti-rabbit immunoglobulin antiserum; HSA, human serum albumin; Med-199, Medium 199; Med-PS, Medium 199 containing penicillin (100 units/ml) and streptomycin (100 μg/ml); NGGG, normal goat gamma globulin; NRS, normal rabbit serum; PHA, phytohemagglutinin; undiluted PHA, PHA (one vial) dissolved in 5 ml of sterile Medium 199.

elsewhere (2). Briefly, the goats were each given three intramuscular injections of rabbit immunoglobulin (100 mg per injection), purified by filtration through a column of Sephadex G-200 and diethylaminoethyl (DEAE) cellulose, in complete Freund's adjuvant at 7-day intervals. The goats were bled 2 wk after the third injection of the antigen. The antiserum contained about 500 mg antibody per ml directed against rabbit gamma globulin, as determined by the quantitative precipitation technique (3).

The complement (C) used was fresh whole guinea pig serum obtained by repeated cardiac puncture of normal, adult guinea pigs. The pooled serum was absorbed with rabbit red cells and kept frozen at −20°C. until used.

Gamma globulin-depleted normal rabbit serum was prepared by precipitating the gamma globulins at 50% saturation with ammonium sulfate. The supernatant, essentially free of gamma globulin by electrophoretic and immunoelectrophoretic analyses, was dialyzed extensively against distilled water, then lyophilized and dissolved in Medium 199 to the appropriate concentration. The pH was adjusted to 7 with sodium bicarbonate.

The rabbits were immunized with human serum albumin (HSA) (Hyland Laboratories, Los Angeles, Calif.). They were injected with HSA (25 mg) via the intravenous route and/or the footpad 1 to 5 months prior to sacrifice. They were sacrificed by i.v. nembutal (50 mg per kg body weight) and the spleen and popliteal lymph nodes were rapidly excised, the entire procedure taking no longer than several minutes. Cell suspensions were prepared as follows. Each organ was cut into small fragments and teased through a sterile wire mesh (50 mesh) by the application of slight pressure into a Petri dish of Med-199 containing penicillin (100 units/ml) (Microbiological Assoc.) and streptomycin (100 μg/ml) (Microbiological Assoc.) (Med-PS). The cells were centrifuged at 800 rpm for 5 min and then suspended to the appropriate cell concentration.

The technique used for cell culture has been described previously (1). The cells were suspended in Med-PS, at a concentration of 2×10^6–8×10^6 cells per ml, to which was added gamma globulin-free normal rabbit serum (NRS), to a concentration of 15%. 4 ml samples of each cell suspension were dispensed into disposable, sterile capped plastic tubes (Falcon Plastics, Los Angeles, Calif.), to which was added the specific stimulating agent. The tubes were then capped and maintained at 37°C for 3, 5, or 7 days. Tritiated thymidine (specific activity, 1 Ci/mmole) was added to each tube 24 hr prior to termination of culture (2 μCi per tube), at which time the tubes were centrifuged at 1500 rpm for 10 min, washed twice with 5% trichloroacetic acid, and digested overnight with Hyamine (Hyamine 10X, Packard Instrument Co., Downers Grove, Ill.). The contents of each tube were then transferred to scintillation counting vials to each of which was added 15 ml scintillation solution (4). The radioactive content of each vial was then determined using the Packard Model 4000 liquid scintillation counter.

Rabbits were subjected to 800 R total body irradiation, using a cobalt 60 source, under the following conditions: skin source distance 200 cm and output 6.97 R/min.

EXPERIMENTAL PROCEDURES AND RESULTS

The Demonstration of an Additive Stimulatory Effect of PHA and Antigen and the Absence of Such an Effect with GARIG and Antigen

Cells of spleen and lymph nodes obtained from rabbits immunized with HSA 4–16 wk prior to sacrifice were suspended in Med-PS to which was added gamma globulin-depleted NRS. 4 ml portions of each of the cell suspensions containing 2×10^6 cells per ml were transferred to the sterile plastic tubes to

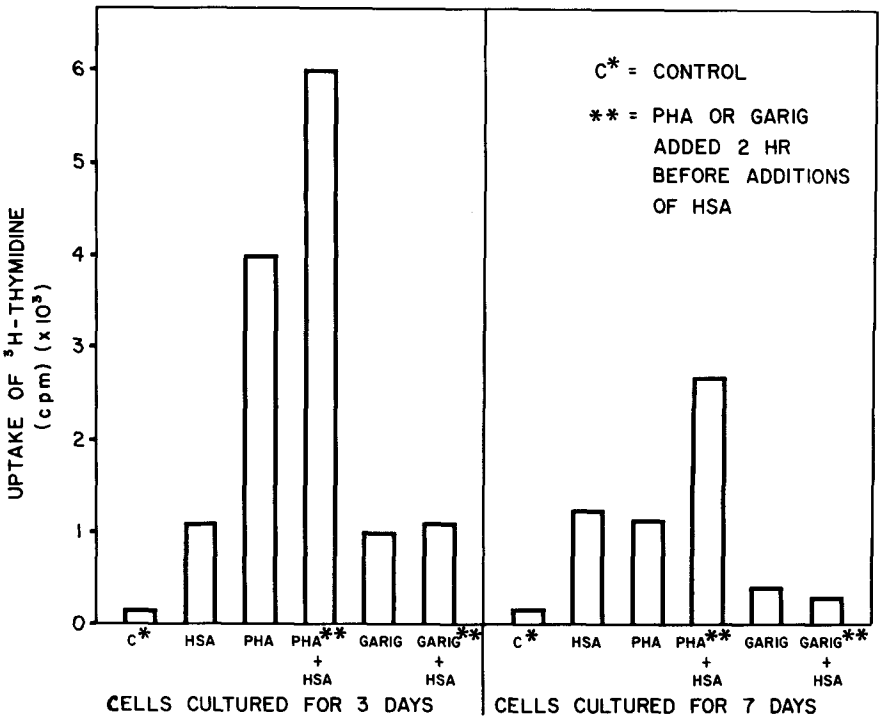

FIG. 1. Incorporation of ^{3}H-thymidine by immune (HSA) rabbit lymph node cells stimulated in vitro with HSA, PHA, GARIG, PHA plus HSA, or GARIG plus HSA.

TABLE I

Incorporation of Tritiated Thymidine by Rabbit Immune (HSA) Lymph Node Cells Stimulated with PHA, HSA, GARIG, PHA Plus HSA, or GARIG Plus HSA

Stimulant added to culture	Mean specific activity* of cultures of immune lymph node cells incubated in vitro for the following periods of time	
	3 days	7 days
PHA	7	2.5
GARIG	4.7	1.2
HSA	3.4	5
PHA + HSA	9.3	7
GARIG + HSA	5	0.9

* The mean specific activity is defined as the ratio of the tritiated thymidine incorporated into the cells in the presence of the stimulus to that incorporated in the absence of the stimulus. The figures presented are based on results of 5 different experiments and represent the mean values.

which were added PHA (0.25 ml, 1:10 dilution), GARIG (0.2 ml) or HSA (5 mg). Some tubes received PHA and HSA or GARIG and HSA. Control tubes received normal goat serum (0.2 ml). The tubes were sealed, incubated for 3 and 7 days at 37°C, and processed for their incorporation of radioactive thymidine as described above.

Incubation of the HSA-immune cells with PHA and HSA resulted in a much greater uptake of tritiated thymidine by the cells than when either stimulant was used alone. The results of a typical experiment are presented graphically

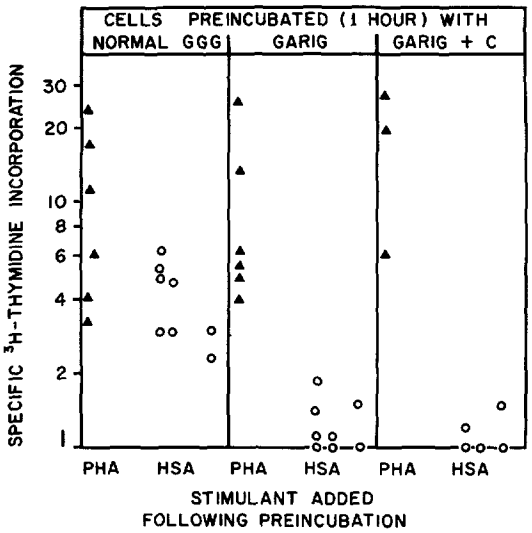

FIG. 2. The inhibition of antigen-induced specific blastogenesis (day 3) of immune rabbit spleen lymphocytes by prior incubation of these cells with GARIG or GARIG and complement.

in Fig. 1 and demonstrate a truly additive stimulatory effect between PHA and HSA. There was no additive stimulatory effect between GARIG and HSA. Moreover, the presence of GARIG completely abolished the stimulatory effect of HSA if the cultures were maintained for 7 days (Fig. 1 and Table I).

The Selective Inhibition by GARIG of Stimulation by HSA of HSA-Immune Rabbit Spleen and Lymph Node Cells In Vitro

Spleen or lymph node cells obtained from rabbits immunized with HSA 4 wk prior to sacrifice were prepared as cell suspensions in Med-PS (8×10^6 cells per ml). 1 ml samples of each cell suspension were dispensed into Falcon plastic tubes and sets of 10 tubes were incubated for 1 hr at 37°C with either GARIG (0.2 ml), GARIG and complement (0.2 ml and 0.25 ml, respectively),

or NGGG (0.2 ml). After incubation, the cells were washed twice with Med-PS and they were resuspended in 4 ml Med-PS-NRS. Of each set of 10 tubes, 2 were kept as controls, two received PHA (0.25 ml, 10-fold dilution), and the remaining 6 tubes received different concentrations of the specific antigen (100 μg–10 mg). The cells were cultured for 3, 5, or 7 days and processed as described above.

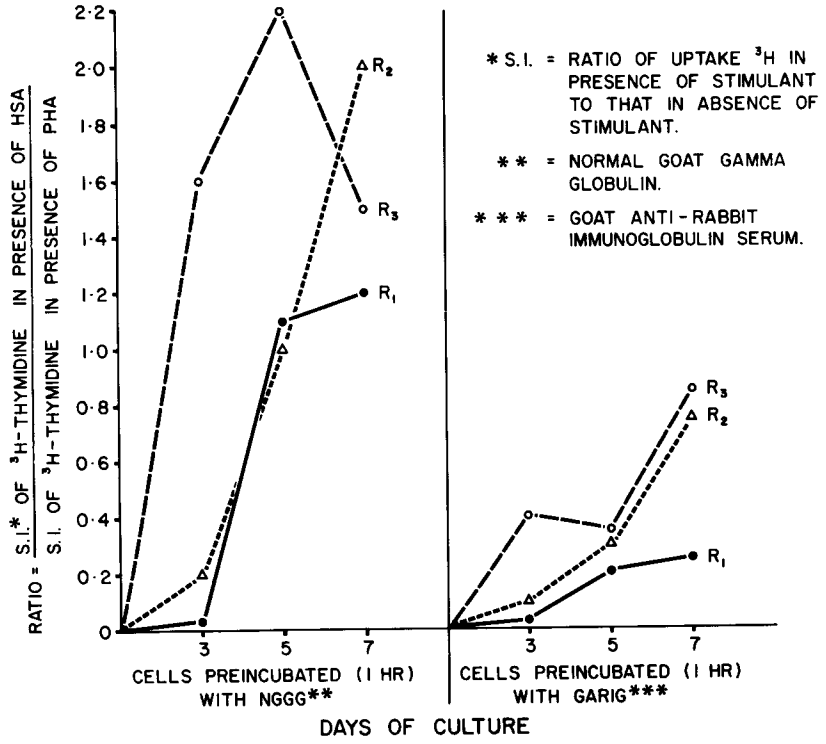

FIG. 3. Uptake of ^{3}H-thymidine by immunized (HSA) rabbit lymph node cells stimulated with PHA or HSA after preincubation for 1 hr with NGGG or GARIG.

Preincubation of the immune cells with NGGG for 1 hr did not affect their subsequent response to HSA or PHA. On the other hand, preincubation of the immune cells with GARIG or GARIG plus complement reduced the specific uptake of tritiated thymidine by the cells subsequently stimulated with HSA without a concommitant effect on their capacity to respond to PHA (Figs. 2 and 3). The inhibition of the capacity to respond to the antigen was almost complete at day 3. The cells showed a tendency to overcome the inhibitory effect of GARIG by days 5 or 7 culture. However, at no time did their response reach the levels attained by control cells preincubated with NGGG (Fig. 3).

Incubation of HSA-Immune Rabbit Spleen and Lymph Node Cells with GARIG, NGGG or Antigen (HSA). Inhibition of the Capacity to Confer Antibody-Forming Capacity to Irradiated (800 R) Immunoincompetent Rabbits (Fig. 4)

Spleen and lymph node cells obtained from rabbits immunized with HSA 4–16 wk prior to sacrifice were prepared as cell suspensions in Med-PS forti-

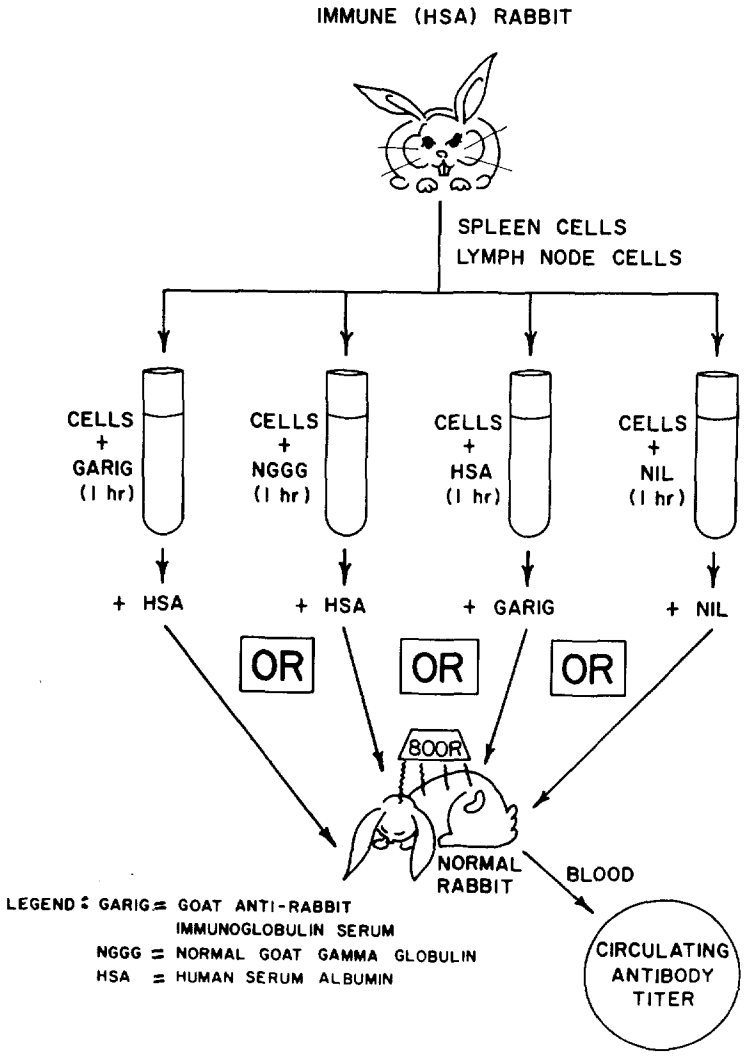

FIG. 4. Protocol for the study of antibody formation in irradiated unimmunized rabbits injected with lymphoid cells preincubated for 1 hr with either NGGG, GARIG or HSA, followed by the addition of HSA or GARIG to the cultures prior to their injection into irradiated hosts.

fied with gamma globulin-free NRS, to a cell concentration of 3×10^6–20×10^6 cells/ml. 1 ml portions of each cell suspension were dispersed into Falcon plastic tubes and sets of 20 tubes were each incubated for 1 hr at 37°C with

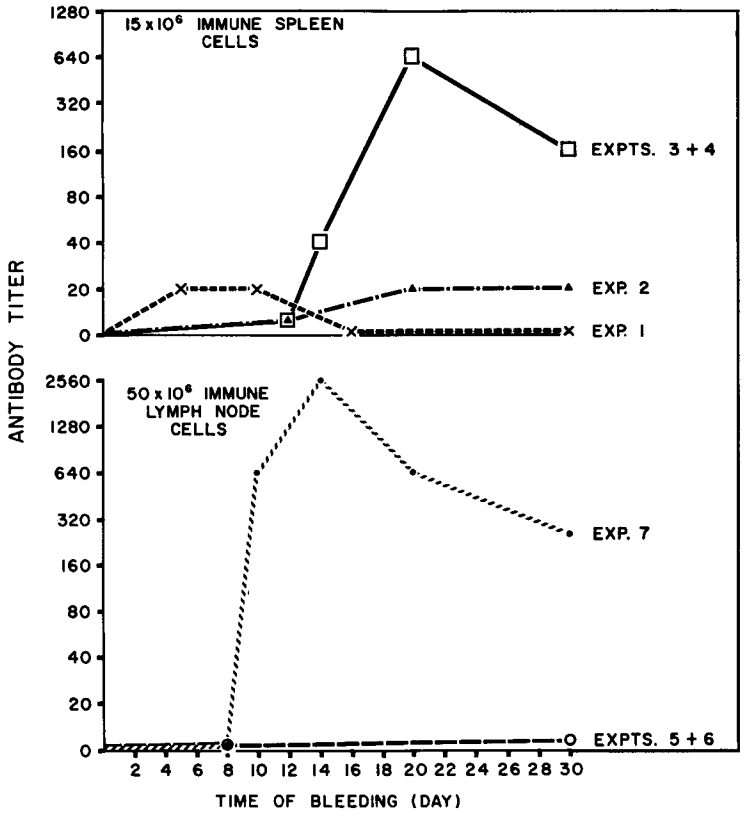

FIG. 5. Antibody formation in irradiated (800 R) rabbits injected with either allogeneic immune spleen cells (15×10^6) or immune lymph node cells (50×10^6) and antigen (HSA). The effect or preincubation of the cells with GARIG, NGGG, or HSA. Expts. 1 and 5: Rabbits injected with cells incubated in medium only. Expts. 2 and 6: Rabbits injected with HSA and cells preincubated for 1 hr with GARIG. Expts. 3 and 7: Rabbits injected with HSA and cells preincubated for 1 hr with NGGG. Expt. 4: Rabbits injected with GARIG and cells preincubated for 1 hr with HSA.

either GARIG (0.2 ml per tube), NGGG (0.2 ml per tube), HSA (1 mg per ube), or nothing (NIL), after which the tubes which had been incubated with GARIG or NGGG each received 1 mg HSA, whereas the tubes which had been ncubated with HSA each received 0.2 ml GARIG. The tubes which had been incubated with NIL did not receive anything. The contents of each set of tubes were then immediately withdrawn and pooled and divided into four

equal parts, each of which was injected into a rabbit which had been subjected to irradiation (800 R) 1–3 hr previously.

Irradiated rabbits which were injected with allogeneic immune cells without antigen or with 15–50 $\times$ 10^6 immune cells preincubated with GARIG for 1 hr and then mixed with the antigen, HSA, produced very little or no circulating antibodies directed to HSA. On the other hand, preincubation of the cells with NGGG did not prevent the production of antibody in recipient irradiated rabbits (Fig. 5). The inhibitory effect of GARIG could be partially overcome when a larger number of immune lymphoid cells (100 $\times$ 10^6 cells) were transferred and the in vitro antigen challenge dose was increased from 10 to 20 mg (Table II). However, the circulating antibody titers of the rabbits which were injected with immune cells preincubated for 1 hr with GARIG were still much lower than those observed in rabbits which were injected with immune cells incubated with NGGG.

TABLE II

Antibody Formation in Irradiated (800 R) Rabbits Injected With Allogeneic Immune (HSA) Lymph Node Cells and Antigen (HSA). Effect of Preincubation of Cells in Vitro with GARIG or NGGG

Cells (100 $\times$ 10^6) preincubated with the following for 1 hr prior to cell transfer	Reagent added to preincubated cells immediately prior to cell transfer	Hemagglutinating antibody titer* on the following days subsequent to cell transfer to irradiated recipient rabbits				
		5	10	14	21	30
NIL	NIL	0	0	0	0	0
GARIG	HSA (20 mg)	0	0	80	160	160
NGGG	HSA (20 mg)	0	160	640	1280	640

* Each value represents the mean of the titers of four different rabbits.

DISCUSSION

A previous investigation from this laboratory was concerned with the demonstration of the heterogeneous nature of the lymphoid cells on the basis of their in vitro responses to a number of mitogenic stimuli—PHA, anti-immunoglobulin antiserum and allogeneic and xenogeneic cells (1). The responses to these stimuli are considered to have immunologic implications in that it was demonstrated that the responses to PHA and anti-immunoglobulin antiserum (GARIG) can be related to the capacity of the lymphoid cells to participate in cellular or humoral immune reactions, respectively (1). Since normal goat serum or normal goat gamma globulin does not possess blastogenic activity, the stimulus provided by GARIG must be attributed to its property as an antiserum directed to rabbit immunoglobulins. It must therefore be presumed that the antibodies in GARIG react with specific immunoglobulin receptor sites on the surface of the lymphocyte, thus triggering off the mitotic cycle.

The mechanism whereby an antigen can stimulate the lymphoid cells of a presensitized individual or animal in vitro to undergo blastogenesis and mitosis is considered to involve the interaction of the antigen with an antibody receptor at the cell surface (5–8). Therefore, it may be postulated that the response of immune cells to stimulation with the immunizing antigen would be interfered with or inhibited entirely by first exposing these immune cells to GARIG. Such proved to be the case. Immune rabbit cells preincubated with GARIG could not respond to subsequent stimulation with the immunizing antigen in vitro. It may be asked why preincubation of the cells with GARIG did not itself result in blastogenesis of the cells. Incubation of rabbit lymphoid cells with GARIG has previously been shown to result in blastogenesis and mitosis after a minimum of 3 days in culture (1). However, the GARIG had to be in contact with the cells for the entire duration of culture in order to facilitate this response (1). In the experiments performed here, immune lymphoid cells were incubated with GARIG for only 1 hr, an incubation time which does not by itself lead to blastogenesis (1). However, these GARIG-incubated HSA-immune cells were incapable of being stimulated by HSA in vitro, unlike immune cells incubated with HSA without any preincubation with GARIG. The cells were not killed by the preincubation with GARIG, since the inhibitory effects of GARIG were as obvious in the absence, as in the present, of complement. The GARIG therefore acts by neutralizing the immunoglobulin receptor sites on the surface of the immune lymphocyte, leaving no free sites to interact with the immunizing antigen.

This interpretation of the results of the in vitro experiments are supported by the cell transfer experiments. Incubation of HSA-immune rabbit spleen or lymph node cells with GARIG prior to incubation of the cells with HSA resulted in the loss of capacity of these cells to transfer antibody-forming capacity to irradiated (800 R) recipients with respect to HSA. On the other hand, immune cells incubated with HSA in vitro followed by incubation with GARIG could transfer immunocompetence to irradiated recipients with respect to HSA to an extent similar to that observed with cells unincubated with GARIG. A similar observation after incubation of immune mouse lymphoid cells with anti-immunoglobulin antiserum has been made by Mitchison (9). These results suggest that GARIG and the specific antigen compete and interact with the same site on the surface of the immune lymphocyte.

In view of the fact that GARIG could inhibit both blastogenesis in vitro and antibody formation by the incubated cells in vivo, the question might logically be asked whether the antigen-induced lymphoblastoid cell is the one engaged in humoral antibody formation. Greaves and Roitt (10) found that a certain proportion of PPD-stimulated immune lymphoid cells could react with fluorescein-conjugated PPD. Similarly, Lamvik (11) demonstrated lytic activity around blast cells in cultures of rabbit immune peripheral lymphocytes incu-

bated with the immunizing antigen (sheep red cells) in agar gel in vitro. On the other hand, Simons and Fitzgerald (12) found no evidence of antibody production by antigen-stimulated lymphoblastoid cells. Grey et al. (13) observed no correlation between the number of cells forming or carrying antibody directed toward sheep red cells (plaque or rosette-forming cells) and those stimulated to transform in vitro by this antigen. Furthermore, Meuwissen et al. (14) have observed that spleen cells of bursectomized and irradiated chickens can undergo normal blastogenesis in vitro in the presence of the specific immunizing antigen, although the bursectomized-irradiated chicken is incapable of synthesizing humoral antibodies. In view of the recent findings of Benezra et al. (15), it would appear that the capacity to respond with blastogenesis and mitosis is indicative of both humoral and cellular immune manifestations. The findings of Davies et al. (16, 17) tend to resolve some of the confusion surrounding the relationship of blastogenesis and antibody formation. They observed that thymic-derived, but not bone marrow-derived, lymphocytes can undergo blastogenesis in response to antigenic stimulation but that it is the nonproliferating bone marrow cells which synthesize antibodies. On the assumption that the peripheral lymphoid organs (spleen and lymph nodes) possess their intrinsic parenchymal populations of lymphocytes as well as thymus-derived and bone marrow-derived cells, the relative numbers of these cells in a cell preparation may be a determining factor as to whether antibody formation will accompany blastogenesis. The results of the experiments carried out in this investigation do not facilitate the resolution of the controversy posed above. However, they unequivocally demonstrate that interaction between a specific cell site on the immune lymphocyte and the immunizing antigen is a necessary prerequisite to permit the cells to transfer antibody-forming capacity to irradiated immunoincompetent recipients.

The finding of an additive stimulatory effect provided by the incubation of the cells with PHA and antigen simultaneously, and the absence of such an effect afforded by GARIG and antigen, lends further support to the thesis of identical receptor sites for GARIG and antigen. These latter results indicate that different cells react to PHA and antigen but that the same cell reacts to GARIG and antigen. In the presence of an optimal stimulus provided by GARIG, the cell cannot be further stimulated by the specific antigen. Further evidence for the correctness of this interpretation is the finding that incubation of the immune lymphoid cells with GARIG plus HSA for 7 days results in, paradoxically, a lesser response than incubation with HSA alone. This finding is probably more apparent than real, however, and is probably due to the fact that cells manifest maximum response by day 3 after stimulation with GARIG, whereas the maximum response to stimulation with antigen is not attained till day 7. Since the response to GARIG is over by day 3, the cells cannot be further stimulated by the specific antigen, thus suggesting that the same cells respond to GARIG and antigen.

An additive effect between PHA and antigenic stimulation has also been observed by other investigators. The simultaneous addition of PHA and sheep red blood cells to cultures of lymphocytes obtained from rabbits previously immunized with sheep red cells induced a blastogenic response greater than that obtained with either PHA or antigen alone (18, 19).

The demonstration of an additive effect between PHA and antigen stimulation brings up the question of the exact significance of the PHA response. Are the cells which respond to stimulation with PHA the same cells which manifest cellular immunity in response to certain types of antigens, such as the trans-

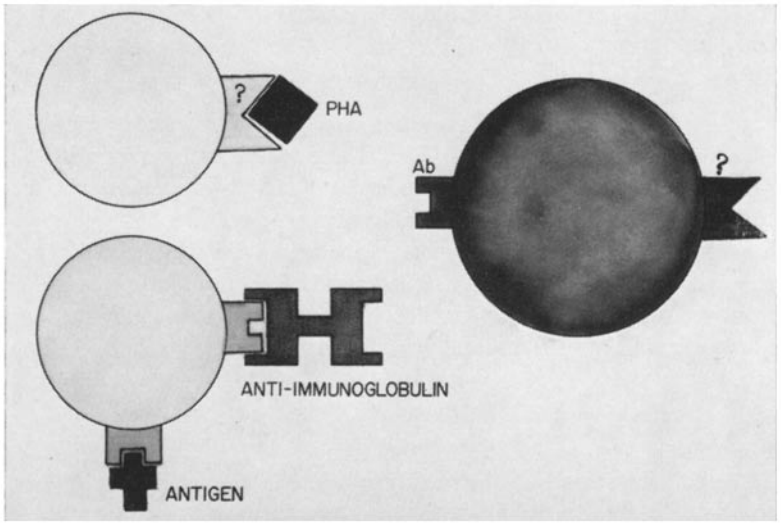

FIG. 6. The existence of a single or two populations of lymphocytes reactive toward PHA, on the one hand, and GARIG and antigen, on the other. A diagramatic representation.

plantation antigens. Clinical and experimental observations indicate that the ability to respond to PHA and participate in reactions of cellular immunity requires an intact functioning thymus (20–23). In a previous communication, it was established that the thymus cells are capable of responding to stimulation with PHA and allogeneic leukocytes, but not to GARIG (1). These responses were considered to be indicative of the capacity to participate in cellular and humoral immune reactions, respectively (1). Furthermore, thymectomy results in the loss of the ability to respond to PHA and allogeneic leukocytes in culture (22). These results suggest that the same cells respond to both PHA and allogeneic leukocytes containing the transplantation antigens. However, two reports (24, 25) have recently appeared, demonstrating a dissociation of in vitro responsiveness to PHA, on the one hand, and allogeneic leukocytes, on the other. Patients were found to consistently respond to one or the other

of these two stimuli. Thus, it would appear that even the cells within the thymic-dependent compartment are themselves functionally heterogeneous in terms of their immunologic functions and that at least two different populations of cells exist, one capable of responding to PHA stimulation and the other to transplantation and other antigens. Both of these responses are, however, considered to represent reactions of cellular immunity.

The results presented in this investigation lend further support to the considerations that there exist two populations of lymphocytes, one capable of responding to PHA and the other capable of responding to stimulation with anti-immunoglobulni antiserum (Fig. 6). These latter cells are also capable of responding to stimulation with common protein antigen, leading to humoral antibody formation.

SUMMARY

There exists in the rabbit a population of lymphocytes carrying immunoglobulin-like receptors on their surface. These receptors interact with antigen and with anti-immunoglobulin antibodies and appear to mediate the recognition process leading to the humoral immune response.

There exists in the rabbit a second population of lymphocytes capable of reacting with phytohemagglutinin. This population of lymphocytes is different from the one capable of reacting with soluble protein antigens or anti-immunoglobulin antiserum and is probably involved in the mediation of cellular immunity.

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