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Antigen-Induced Suppression of Antibody Synthesis during an *in Vitro* Secondary Response (40559)

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Antigenic competition appears to be of importance in immune responses in nature and is of potential significance in studying the regulation of immune responses. Most of the early work was performed *in vivo* and reviewed by Pross and Eidenger (1), and Liacopoulos and Ben-Efraim (2). Radovich and Talmage (3) suggested that competition was not involved when they observed less suppression of antibody formation in subjects immunized simultaneously with two antigens than if one antigen was injected before the other. These and similar findings led Pross (4) to introduce the term "antigen-induced suppression."

In vitro studies of antigen-induced suppression of antibody production can be classified into three categories: 1. Suppression of an *in vitro* primary response in subjects previously exposed to a heterologous antigenic stimulus *in vivo* (4–7); 2. suppression of an *in vitro* primary response during an *in vitro* secondary response to a heterologous antigen (8–10); and 3. suppression of an *in vitro* secondary response with supernatants from cell cultures undergoing a secondary response to a heterologous antigen (11). In most of these studies, plaque assay techniques were used and soluble factors were implicated as causing the antigen-induced suppression.

In the present study, rabbits were injected in the foot pads with two or more priming antigens 4 to 8 weeks prior to preparing cultures from the draining lymph nodes. Aliquots of each cell suspension were cultured in the presence or absence of each of the priming antigens; after which, the spontaneous antibody production, the anamnestic responses, and antigen-induced suppression occurring in these cultures were quantitatively determined by means of a solid-phase radioassay.

Materials and methods. Antigens. Commercial preparations of the following antigens were used in this study: human serum albumin (HSA), rabbit serum albumin (RSA), and horse γ-globulin (HoGG) (United States Biochemical Corp.), bovine serum albumin (BSA) (Calbiochem), porcine serum albumin (PSA) (Nutritional Biochemicals Corp.), and horseshoe crab hemocyanin (HCH) (Worthington Biochemical Corp.).

Immunization. Antigens used for immunization were dissolved (10 mg/ml) in phosphate buffer (0.2 *M*, pH 7.2) and aggregated with heat at 70° for 15 min. Solutions of aggregated antigens were mixed with each other and diluted with physiological saline solution to a concentration of 2 mg/ml for each antigen. An emulsion was prepared by vigorously mixing equal volumes of the antigen mixture and Freund's complete adjuvant (Difco Laboratories). New Zealand white rabbits of either sex weighing 6 to 8 lbs were injected in each foot pad with 0.5 ml of the antigen–adjuvant emulsion. After 4 to 8 weeks, the rabbits were sacrificed and cell cultures prepared from the draining lymph nodes.

Cell cultures. Lymphoid cell suspensions (2×10^7 cells/ml) were prepared and cultured as described by Herskowitz and Stavitsky (12). Prior to culture, aliquots of the cell suspension were rinsed in Hanks' balanced salt solution and exposed to a single aggregated antigen (10 μg/ml of suspension). After 15-min contact with antigen, the cell suspensions were centrifuged and the supernatant replaced with culture media. (The culture media consisted of minimum essential medium (Eagle) (Grand Island Biological Co.) containing 10% normal rabbit serum and other components as described by Herskowitz and Stavitsky (12).) The control to measure the spontaneous antibody response consisted of a portion of the cell suspension not exposed to antigens *in vitro*. After 3 days at 37°, the nonradioactive culture fluid was replaced with media containing 0.5 μCi of uniformly labeled L-[^{14}C]leucine (250–300 mCi/m*M*, ICN) per milliliter. After 3-day incubation in radioactive culture media, the cell culture

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fluid was collected and assayed for ^{14}C-labeled antibody.

Measurement of labeled antibodies. The amount of radioactive antibody in the cell culture fluids was measured according to the methods developed by E. J. Green, J. G. Tew, and G. A. Miller (unpublished results). Antigen-coated plastic vials were prepared by allowing Bio-Vials (Beckman Instruments) filled with a solution of 1 g/liter each of antigen and of 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (Story Chemical Co.) in sodium bicarbonate-deficient Hank's solution, to stand for 1 hr at room temperature. The vials were washed 18 times with tap water, air dried at room temperature overnight, and capped. These antigen-coated vials could be stored at 4° for months without loss of antigenicity. The antibody assay was performed by a technique using paired antigen-coated vials. One vial was filled with 0.4 ml of the cell culture fluid containing radioactive antibody, 0.1 ml of 0.5% sodium azide, and 2.4 ml of physiological saline solution. The other was filled as above except that 1 mg of soluble antigen was added to the vial prior to the addition of the other solutions. Both vials were placed on a rotary shaker at room temperature overnight before the fluid was poured off and the vials washed 14 times with tap water. The vials were then filled with Aquasol (New England Nuclear) and counted 7 hr later in a Beckman LCS-150 liquid scintillation counter. The radioactivity in the first vial represented the total radioactivity bound. The binding in the second vial was assumed to be nonspecific since specific antibody binding would be blocked by the excessive amount of free antigen. A measure of the specific binding of labeled antibody to the antigen on the vial surface was obtained by subtracting the nonspecific binding from the total count. This was termed the specific count and expressed as cpm/ml of culture fluid.

Results. In order to determine whether the cross-reactivity of the priming antigens observed in this system would be comparable to that detected in other systems, specific binding of radioactively labeled anti-HSA to various antigen-coated vials was measured. This anti-HSA was obtained from the cultures of lymphoid cells from rabbits immunized with HSA only. As shown in Table I, HSA exhibits moderate cross-reactivity with BSA and PSA, less with HoGG, and none with HCH. Rabbit serum albumin (RSA)- and HSA-coated vials were used as negative and 100% controls, respectively. As will be shown later, the relatedness of priming antigens is an important factor in determining the type of antibody responses that follow *in vitro* exposure to antigen.

TABLE I. REACTION OF LABELED ANTI-HSA WITH VIALS COATED WITH DIFFERENT ANTIGENS[a]

Antigen coating	cpm[b]	Percentage cross-reactivity with HSA
HSA	5288	100
BSA	688	13
PSA	300	6
HoGG	105	2
HCH	0	0
RSA	0	—

[a] Antigen-coated vials were treated with pooled culture fluid from rabbits immunized with HSA only.
[b] Specific cpm per ml of culture fluid.

In the next experiments, rabbits were immunized in two groups. Each rabbit in one group received a priming injection of HSA, BSA, PSA, and HoGG. The other rabbits received HSA, HoGG, and HCH. As indicated above, the three albumin antigens in the first group are moderately cross-reactive but the other antigen (HoGG) is only weakly related. The three immunizing antigens in the second group are nearly or completely unrelated to each other. Portions of the cell cultures of lymph nodes were separately exposed *in vitro* to a single antigen. The effects of exposure to each priming antigen on antibody responses to all priming antigens were measured and compared to the spontaneous responses that take place in the absence of *in vitro* antigen exposure.

The spontaneous responses recorded on the first line of both Tables II and III occurred without an *in vitro* antigenic stimulation and varied with the different priming antigens. With both groups, the spontaneous production of antibody by the cell cultures was greatest against HSA.

When the spontaneous response was compared with the results following *in vitro* exposure to the homologous antigen, it was obvious that specific anamnestic responses occurred following *in vitro* exposure to single priming antigens. With one exception, *in vitro* exposure of the cell cultures to a priming

TABLE II. ANTIBODY RESPONSES FOLLOWING *IN VITRO* STIMULATION OF CELL CULTURES FROM RABBITS PRIMED WITH HSA, BSA, PSA, AND HoGG[a]

Antigen exposure	Binding of labeled antibody							
	Anti-HSA		Anti-BSA		Anti-PSA		Anti-HoGG	
	cpm[b]	*P*[c]	cpm	*P*	cpm	*P*	cpm	*P*
None[d]	3398		1703		1380		664	
HSA	5941	<0.05	2340	<0.005	1923	<0.05	445	<0.025
BSA	4243	>0.2	4286	<0.05	2235	>0.1	417	<0.005
PSA	2333	>0.2	2092	>0.2	3422	>0.1	388	<0.005
HoGG	1233	<0.005	803	<0.005	578	<0.005	4092	<0.001

[a] Cumulative results from five rabbits.
[b] Mean specific cpm per ml of culture fluid.
[c] Obtained from paired *t* test on percentage change from spontaneous responses.
[d] Spontaneous response.

TABLE III. ANTIBODY RESPONSES FOLLOWING *IN VITRO* STIMULATION OF CELL CULTURES FROM RABBITS PRIMED WITH HSA, HoGG, AND HCH[a]

Antigen exposure	Binding of labeled antibody					
	Anti-HSA		Anti-HCH		Anti-HoGG	
	cpm	*P*	cpm	*P*	cpm	*P*
None	18900		950		920	
HSA	28900	>0.2	280	<0.05	270	<0.01
HCH	4900	<0.01	16200	<0.05	220	<0.01
HoGG	8700	<0.02	150	<0.01	12300	<0.02

[a] Cumulative results from four rabbits.

albumin generally enhanced the antibody responses to the other priming albumins (Table II). In contrast, this exposure to any of the three priming albumin antigens significantly decreased production of antibody against HoGG below that which was spontaneously produced in other cultures. Likewise, *in vitro* exposure of cell cultures to HoGG significantly depressed antibody production against all three types of albumin antigens. These changes are graphically illustrated in Fig. 1 in which the enhancement or suppression of the antibody response was calculated as percentage changes of the spontaneous response.

The results of similar experiments in which three non-cross-reacting antigens were used are recorded in Table III. In every case, the *in vitro* exposure of cell cultures to a single antigen enhanced production of homologous antibody but significantly suppressed the antibody production against the other two priming antigens. The percentage changes, with respect to the spontaneous responses, following *in vitro* exposure to single antigens in this experiment are represented in Fig. 2.

In an effort to determine the dependence of suppression upon previous exposure to the *in vitro*-stimulating antigen, two rabbits were injected with only two (HSA and HoGG) of the three antigens used above and their cell cultures exposed separately to all three of the unrelated antigens. As expected from results recorded in Table II and Fig. 2, HSA inhibited anti-HoGG production and vice versa. In contrast, exposure of cell cultures to an unrelated antigen that was not used for immunization (HCH) did not suppress the spontaneous antibody responses to immunizing antigens.

In order to investigate the possible involvement of antibody feedback inhibition in suppression, the following experiment was performed. Lymphoid cells from a single rabbit immunized with HSA, HGG, and HCH were cultured in two groups. Each group of cells was divided into portions and exposed separately to each of the priming antigens as in previous experiments. One group was cultured as usual in medium containing normal rabbit serum. The other group was cultured in medium containing rabbit anti-HSA antisera for the first 3 days of culture. After the first 3 days, the fluid was poured off and both groups were treated as prescribed by the nor-

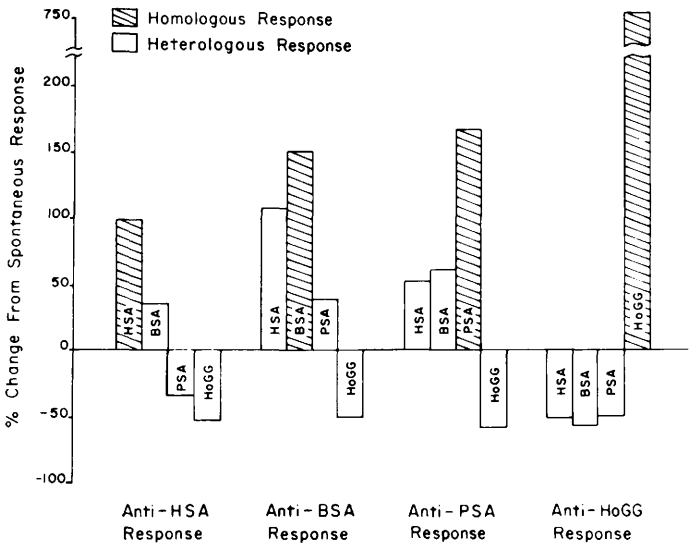

FIG. 1. Enhancement and suppression of antibody responses to HSA, BSA, PSA, and HoGG in lymphoid cell cultures following *in vitro* exposure to each of the priming antigens. Cell cultures were prepared from the draining lymph nodes of rabbits previously immunized with HSA, BSA, PSA, and HoGG. The antigens to which the cell cultures were exposed *in vitro* are designated on each bar.

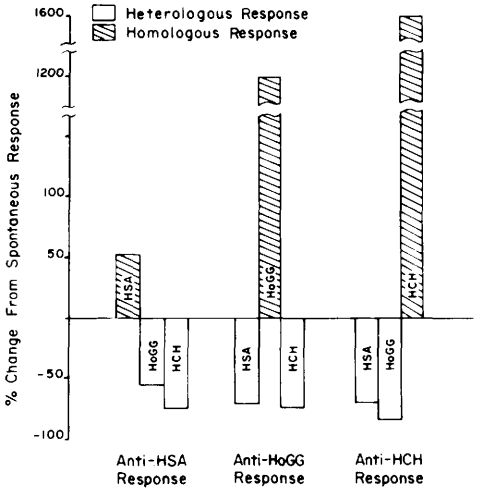

FIG. 2. Enhancement and suppression of antibody responses to HSA, HoGG, and HCH in lymphoid cell cultures following *in vitro* exposure to each of the priming antigens. Cell cultures were prepared from the draining lymph nodes of rabbits previously immunized with HSA, HoGG, and HCH. The antigens to which the cell cultures were exposed *in vitro* are designated on each bar.

mal culture procedure. The presence of anti-HSA in the culture fluid suppressed the spontaneous anti-HSA response by 97% and the anamnestic anti-HSA response by 90%, but did not suppress the spontaneous or anamnestic responses to the other unrelated priming antigens (HoGG and HCH).

Enhancement of responses by *in vitro* exposure of cells to a cross-reactive antigen could be either determinant or molecule specific. In other words, the observed enhancement could be a result of increased production of antibody against antigenic determinants on the heterologous antigen which are similar to or identical with those contained on the antigen to which the cells are exposed; or, production of antibody against all antigenic determinants on the heterologous molecule might be enhanced. In order to determine which possibility occurred, the following experiment was performed. Culture fluid which remained from the experiment recorded in Table II and Fig. 1 was used. This fluid was obtained from cultures exposed *in vitro* to each of the albumins used for immunization (HSA, BSA, and PSA) as well as from unexposed cultures. Prior to the addition of pooled culture fluid from each exposure group, 1 mg of soluble inhibitor (HSA, BSA, or PSA) was added to PSA-coated vials. Another PSA-coated vial was filled with culture fluid from each exposure group without addition of antigen and served as a control. Except for the addition of soluble antigens to the assay vials, this assay was performed as in previous assays. Inhibition by each antigen of binding to PSA-coated vials was determined by subtracting the amount of binding

which occurred in the vials containing each soluble antigen from the binding which occurred in the absence of inhibition. (The values for inhibition by PSA are equivalent to what was previously termed as specific anti-PSA binding in other experiments.) Percentage inhibition was calculated by designating inhibition by PSA as 100%. The results obtained are represented in Table IV. Three basic conclusions are inferred by this data: 1. Very little antibody was present in the fluid from any of the cultures which was specific for PSA determinants not cross-reactive with HSA or BSA except in those exposed to PSA *in vitro*; 2. enhancement of binding to PSA in fluid from cells exposed to HSA or BSA *in vitro* occurred only to antigenic determinants cross-reactive with those on the antigen to which the cells were exposed; 3. exposure of cells to HSA or BSA resulted in a shift of the PSA-binding antibody population toward greater specificity for the antigen determinants to which the cells were exposed.

Discussion. Herscowitz and Stavitsky (12) demonstrated that cells from the draining lymph nodes of rabbits previously primed with a protein antigen will spontaneously produce radioactive antibody when cultured *in vitro* with L-[^{14}C]leucine. As confirmed in this study, this spontaneous antibody response was shown to be enhanced by an *in vitro* reexposure to the priming antigen and suppressed by the addition of homologous antibody to the culture fluid (13). It was postulated by Tew *et al.* (13, 14) that this spontaneous antibody production is in reality an anamnestic response to cell-bound antigen. They suggest that this spontaneous anamnestic response might be induced by the removal of antibodies which would otherwise mask this cell-bound antigen. Tew *et al.* (14) showed that this antigen remained associated with accessory cells for months after immunization. Consequently, this study would involve interactions between anamnestic responses to cell-bound antigens as well as to antigens added to the cell cultures *in vitro*. The observation that exposure of these cell cultures to microgram quantities of antigen increased antibody production indicated that if the spontaneous response is an anamnestic one, the cell-bound antigens were not presented under ideal conditions for an optimal secondary response.

TABLE IV. INHIBITION OF BINDING OF CELL CULTURE FLUID FROM A RABBIT PRIMED WITH HSA, BSA, PSA, AND HoGG TO PSA-COATED VIALS BY HSA, BSA, AND PSA

Antigen exposure	Soluble inhibitor[a]	Binding (cpm)	Inhibition[b]	
			cpm	%
None	None	964		
	HSA	338	626	83
	BSA	332	631	84
	PSA	213	751	100
HSA	None	1408		
	HSA	376	1032	95
	BSA	828	580	53
	PSA	316	1091	100
BSA	None	2022		
	HSA	860	1162	70
	BSA	429	1593	96
	PSA	358	1664	100
PSA	None	2202		
	HSA	1074	1120	62
	BSA	1718	485	26
	PSA	371	1832	100

[a] Absorption was performed by adding 1 mg of antigen in soluble form to the PSA-coated vials prior to the addition of culture fluid.

[b] Inhibition values were obtained by subtracting binding to vials which contained absorbing antigen from the binding which occurred in the absence of absorbing antigen.

In addition to the anamnestic response to homologous antigens observed in this study, enhancement of antibody production by highly cross-reactive antigens usually occurred. These increases were never as great as the specific anamnestic responses. This stimulation between cross-reactive antigens was shown by absorption to be due to increased production of antibody against common antigenic determinants. This study also showed that cells from rabbits primed with HSA, BSA, PSA, and HoGG produced very little anti-PSA antibody which did not cross-react with HSA or BSA except those exposed to PSA *in vitro*. This would indicate that the spontaneous response observed against PSA is probably largely due to a carry-over from the spontaneous responses to HSA and BSA. It is not known why the spontaneous response to PSA determinants not common to HSA or BSA was so low. It is possible that responses to these determinants were suppressed without the *in vitro* addition of HSA or BSA. Nevertheless, suppression of responses to these determinants upon exposure of the cells

to HSA or BSA could not be observed since no appreciable spontaneous response occurred to these determinants.

Preliminary attempts have been made to elucidate the mechanisms responsible for the suppression of antibody production by *in vitro* exposure to unrelated priming antigens observed in this study. This suppression was not induced by *in vitro* exposure to a nonpriming antigen. This indicates that the suppression was not directly related to the antigen but was induced by conditions which follow a second exposure to a priming antigen. As previously seen by Tew *et al.* (13), it was observed in this system that suppression due to antibody feedback is very specific. Consequently, the antibody produced during an anamnestic response to one antigen could not account for the suppression of antibody production against other unrelated antigens. Soluble factors other than antigen and antibody have been implicated (4–6, 8–11) as the cause of *in vitro* antigen-induced suppression. A type of suppression which might involve mechanisms similar to antigen-induced suppression was reported in studies (15–17) in which soluble factors from concanavalin-A-stimulated thymus-derived lymphocytes suppressed antibody production. Some investigators (5, 8–10) have implied that thymus-derived suppressor lymphocytes may well be the source of suppressor factors involved in antigen-induced suppression of antibody production. In contrast, Schrader (7) was able to restore the antibody response to "antigen suppressed" spleen cells by trypsinization and by addition of the anti-θ treated peritoneal exudate cells from normal animals, and maintained that macrophages were the site of antigenic competition. During the course of the present study, a number of attempts to demonstrate the existence of either soluble suppressor factors or suppressor cells in our cell cultures were unsuccessful. Consequently, the mechanism of the reported *in vitro* suppression is still undetermined.

This study made use of a system for inducing and measuring antibody production not previously applied to investigations on antigen-induced suppression. This system is well suited for such a study because production of radioactive antibody under carefully controlled conditions can be measured with relative ease in a highly sensitive and quantitative fashion.

Summary. The production of antibodies in cell cultures from the draining lymph nodes of rabbits injected in the foot pads with two to four different protein antigens was studied. Antibodies against all of the immunizing antigens were produced simultaneously when aliquots of the cells were cultured 4 to 8 weeks after immunization in the absence of further antigen exposure. *In vitro* exposure to a single immunizing antigen always resulted in an anamnestic response to the added antigen and a general enhancement of antibody production against cross-reactive determinants on other immunizing antigens. However, antibody production against unrelated immunizing antigens was always significantly suppressed by this exposure. This suppression was not the result of antibody feedback and could not be induced unless the unrelated antigen had been used as a priming antigen.

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