

CRITICAL ROLE OF DETERMINANT PRESENTATION IN THE  
INDUCTION OF SPECIFIC RESPONSES IN IMMUNO-  
COMPETENT LYMPHOCYTES\*

BY DAVID H. KATZ AND EMIL R. UNANUE‡

(From the Department of Pathology, Harvard Medical School,  
Boston, Massachusetts 02115)

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The introduction of antigen sets in motion a series of cellular events that culminate in a specific immune response. The nature of the response induced may be manifested either positively, i.e. by immunity, or negatively, i.e. by tolerance, but nevertheless always in a highly specific fashion. We now recognize that the specificity of the response reflects the respective functions of both thymus-derived (T)<sup>1</sup> and bone marrow-derived (B) lymphocytes. In the case of humoral immune responses, the concomitant participation of T and B lymphocytes is required for optimal immunity to most antigens (reviewed in reference 1).

Numerous experimental observations made during the past decade have given us some understanding of the mechanisms by which an antigen molecule induces a state of tolerance or immunity. It is clear that the molecular form in which the antigen is presented is crucial for effective triggering in that some forms of antigen, principally those taken up poorly by the mononuclear phagocyte system, induce tolerance whereas the opposite effect, i.e. immunity, can be obtained with appropriate forms of antigen that are readily concentrated by macrophages (reviewed in reference 2). These observations have called attention to the macrophage as an effective accessory cell that favors positive induction of immunity. Indeed, it is now well known through a variety of direct experimental approaches that antigen bound to macrophages is in a form that favors triggering and, moreover, that such an effective form of antigen presentation can be blocked in part by the introduction of antigen not bound to macrophages (3). Macrophage-associated antigen can trigger both T and B lymphocytes: (a) macrophage-bound antigen elicits cell-mediated immunity (4, 5); (b) thymus deprivation abrogates the response to macrophage-bound antigen (6); and (c) hapten-specific

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<sup>1</sup> *Abbreviations used in this paper:* B cells, bone marrow-derived lymphocytes; BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; ECDI, water-soluble carbodiimide; FCS, fetal calf serum; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; MEM, minimum essential medium; M $\phi$ , macrophage; NA, nonadherent; PFC, plaque-forming cells; RAMG, rabbit antimouse immunoglobulin antibodies; SRBC, sheep erythrocytes; T cells, thymus-derived lymphocytes; TNP, 2,4,6-trinitrobenzenesulfonic acid.

secondary responses, which require the participation of T and B cells, can be elicited by hapten-protein conjugates bound to macrophages (7, 8). The available *in vivo* evidence could explain the role of macrophages in immune induction on the basis of its capacity both to take up and concentrate extracellular antigen (thereby reducing the pool of potentially tolerogenic molecules) and to present the antigen for effective T and B cell interactions (2). It is altogether clear that macrophages do not determine the specificity of the immune response; moreover, there is strong, suggestive evidence from *in vitro* studies that the extent to which these accessory cells are needed may depend upon the nature of the antigen employed (2).

It has become increasingly evident that we must have a more precise understanding of where and how the macrophage fits into the sequential cellular events that follow antigen administration. Critical questions in this regard concern: (a) whether antigen presentation by macrophages is merely a better way or, in fact, a necessary way to effect triggering of specific lymphocytes; (b) whether if antigen presentation by macrophages is necessary, this is true for both T and B cells, T cells alone, or B cells alone; (c) whether or not there is something unique about the macrophage as an antigen-handling cell; (d) whether the capacity of macrophages to present antigen so effectively is determined to any significant extent by the presence of antibody molecules specific for the antigenic determinants; and finally, (e) the exact mechanism by which macrophage antigen presentation provides an effective pathway for immune induction. Is the macrophage simply serving as a cohesive focal point that brings together the few committed T and B cells? Or, alternatively, is the macrophage playing some other role perhaps related to the molecular display of antigen molecules on its surface, or to other unknown events?

In the present study, we have attempted to answer some of these questions by utilizing *in vitro* systems to functionally assess the role of macrophage-bound hapten-protein conjugates. The results of these experiments indicate a critical role for determinant presentation on cell surface membranes in the process of signal transmission in specific immunocompetent lymphocytes. Moreover, an argument is presented, on the basis of these data, for the concept that lymphocyte triggering by macrophage-bound antigen initiates, rather than intervenes in, the sequence of cellular events in physiologic T and B cell interaction.

#### *Materials and Methods*

*Proteins and Hapten-Carrier Conjugates.*—The following 2,4-dinitrophenyl (DNP) conjugates were prepared as previously described (9, 10): DNP<sub>32</sub>-bovine gamma globulin (BGG), DNP<sub>43</sub>-BGG (subscript refers to average number of moles of DNP per mole of carrier), and DNP<sub>9</sub>-keyhole limpet hemocyanin (KLH) (subscript refers to average number of moles of DNP per 100,000 mol wt units of KLH).

*Animals and Immunizations.*—BALB/c and A/J mice of either sex were obtained from the Jackson Laboratory, Bar Harbor, Maine. A/St mice were purchased from West Seneca Laboratories, Buffalo, N. Y. All mice were immunized at 2–3 mo of age intraperitoneally with 100  $\mu$ g of either DNP-KLH or DNP-BGG emulsified in complete Freund's adjuvant (CFA) and used at various times (1–4 mo) thereafter.

*Preparation of Antigen-Bearing Cells.*—

*Macrophages:* Mice were injected intraperitoneally with 1.0 ml of 10% proteose peptone (Difco Laboratories, Detroit, Mich.) and killed 3 days later. Cells were isolated, aseptically,

from the peritoneal cavity of such mice using minimum essential medium (MEM) (Eagle) containing 10 U/ml of heparin and 5% fetal calf serum (FCS). This procedure routinely yielded from normal mice about  $8-15 \times 10^6$  cells consisting of 85% typical macrophages. In some experiments the cells were isolated from peptone-injected mice given 660 R of whole-body X irradiation 24 h before harvesting. The average number of cells from such irradiated donors was  $4 \times 10^6$ , consisting of more than 98% typical macrophages.

*Fibroblasts:* Mouse embryo fibroblasts were isolated from 14-day-old embryos by trypsinization. Cells were maintained in culture in MEM with 5% FCS; every 3-4 days the cell monolayer was trypsinized and transferred to fresh culture dishes. Before use in the experiments to be described, the cells were harvested from the dishes by trypsinization and were washed with fresh MEM.

Suspensions of macrophages or fibroblasts in MEM containing 5% FCS and devoid of any mouse serum proteins were incubated with antigens for 30 min, usually on ice. The cells were then washed four times and resuspended to the appropriate concentration. In many experiments antigen uptake was quantitated by using antigen labeled with  $^{125}\text{I}$  by a chloramine T method (specific activity around  $1 \mu\text{Ci}/\mu\text{g}$ ) (11).

In most experiments,  $2 \times 10^6$  macrophages were exposed to  $4 \mu\text{g}$  of DNP-KLH (in a total volume of 1.4 ml) for 30 min on ice, washed thoroughly, and then resuspended in 2.0 ml of culture medium ( $1 \times 10^6$  cells/ml). Under these conditions the percent uptake of DNP-KLH ranged from 0.25 to 0.31. Since the resulting macrophage suspension was distributed among four culture dishes ( $5 \times 10^5$  cells/dish), the amount of macrophage-bound DNP-KLH per culture ranged from 2.5 to 3.1 ng. Fibroblasts were used at 10-fold lower amounts per culture ( $5 \times 10^4$ ) than in most experiments performed with macrophages alone. A comparison was therefore made of the uptake of various concentrations of DNP-KLH ( $0.04-40.0 \mu\text{g}/2 \times 10^5$  cells) by macrophages and fibroblasts, according to techniques previously described (12). At a given concentration of DNP-KLH, uptake was two- to three-fold higher by macrophages than fibroblasts.

*Spleen Cell Cultures.*—The Mishell-Dutton culture system was employed, reagents and conditions being as previously described (13, 14). Cultures were established at spleen cell densities of  $12 \times 10^6$  cells/ml. After 4 days of incubation, cells from individual dishes of triplicate cultures were assayed, by a modification of the hemolytic plaque technique (13, 15, 16), for IgM and IgG anti-DNP antibody-producing plaque-forming cells (PFC). Sheep erythrocytes (SRBC) lightly conjugated with 2,4,6-trinitrobenzenesulfonic acid (TNP) were used as indicator (17). IgG PFC were developed using a rabbit antimouse immunoglobulin-facilitating serum.

*Measurement of Anti-DNP Antibodies.*—Serum anti-DNP antibody levels in micrograms per milliliter were determined by a modified Farr technique (18, 19) using [ $^3\text{H}$ ]DNP- $\epsilon$ -amino-*N*-caproic acid and standard curves as previously described (10).

*Statistical Analysis.*—The number of PFC and serum antibody titers were logarithmically transformed, and means and standard errors were calculated. Group comparisons were made employing Student's *t* test.

*Miscellaneous.*—The isolation procedures and specificity of the rabbit antimouse immunoglobulin (Ig) antibodies (RAMG) employed and the method of immunohistochemical quantitation of B lymphocytes have been described (20).

## RESULTS

### *Immunogenicity and Specificity of Macrophage-Bound DNP-Carrier Conjugates.*—

*In vitro secondary anti-DNP antibody responses of primed spleen cells to soluble antigen:* Before testing the immunogenicity of macrophage-bound DNP proteins, initial experiments were designed to establish conditions for obtaining

reproducible secondary anti-DNP antibody responses to soluble DNP-KLH in vitro. A dose-response experiment with spleen cells from primed BALB/c and A/St mice is illustrated in Fig. 1. Several points of importance must be emphasized. First, the in vitro secondary anti-DNP antibody responses of BALB/c mouse spleen cells were consistently higher than those of A strain mice.

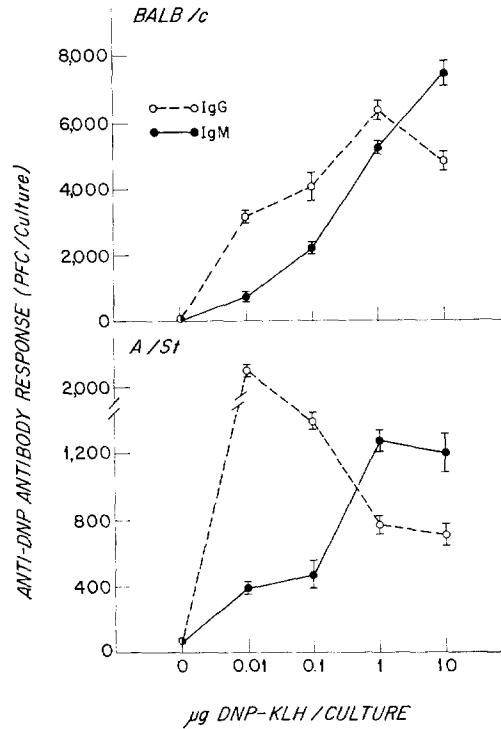


FIG. 1. In vitro secondary anti-DNP antibody responses of primed spleen cells to soluble antigen. Spleen cells from BALB/c and A/St mice primed with DNP-KLH 90 and 63 days, respectively, before sacrifice were cultured for 4 days in the presence of varying doses of soluble DNP-KLH (0.01–10.0  $\mu\text{g}/\text{culture}$ ). The IgM and IgG anti-DNP PFC responses obtained at each dose are expressed as geometric means of three cultures. Vertical bars represent the range of standard errors.

Second, the IgM and IgG responses did not necessarily parallel one another, but varied, depending on the amount of antigen in the culture. Moreover, the level of response of a given antibody class varied with time after priming as well as with dose of in vitro antigen, a point established previously by Bullock and Rittenberg (21). Although consistently higher in vitro responses were obtained with spleen cells from BALB/c mice, most experiments in this report were performed with A/St cells, since it is with this strain that most previous studies on antigen handling by macrophages have been performed.

*In vitro* secondary anti-DNP antibody responses to macrophage-bound DNP proteins: Spleen cells from DNP-KLH-primed A/St mice were cultured with and without soluble DNP-KLH (1  $\mu\text{g}/\text{ml}$ ) and either with  $5 \times 10^5$  normal syngeneic macrophages or  $5 \times 10^5$  DNP-KLH macrophages. In this experiment, the total amount of DNP-KLH on  $5 \times 10^5$  macrophages per culture dish was 0.003  $\mu\text{g}$ . The results are summarized in Table I. Those cultures in which no macrophages were added (group A) developed slightly higher IgM than IgG secondary anti-DNP responses to soluble DNP-KLH. The addition of normal macrophages (group B) caused an appreciable, but not significant, increase in the IgM background but, more impressively, a two- to three-fold increase in

TABLE I  
*Effect of Normal and DNP-KLH-Bearing Macrophages on In Vitro Secondary Anti-DNP Antibody Responses*

Protocol*			Anti-DNP PFC/culture†			
Cells cultured	Group	Macrophages	No soluble antigen		1 $\mu\text{g}$ soluble DNP-KLH	
			IgM	IgG	IgM	IgG
12 $\times$ 10 <sup>6</sup> DNP-KLH-primed A/St spleen cells	A	None	93	53	1,024	825
	B	5 $\times$ 10 <sup>5</sup> normal M $\phi$	213	53	3,125	1,756
	C	5 $\times$ 10 <sup>5</sup> DNP-KLH M $\phi$	1,663	2,234	—	—

\* Spleen cells from A/St mice primed 4 mo earlier with DNP-KLH were cultured for 4 days with (far right column) or without (second column from the right) soluble in vitro immunogen (1  $\mu\text{g}/\text{ml}$  DNP-KLH) and either with no added macrophages (M $\phi$ ) (group A),  $5 \times 10^5$  normal M $\phi$  (group B), or  $5 \times 10^5$  DNP-KLH M $\phi$  (group C). The total amount of DNP-KLH on  $5 \times 10^5$  M $\phi$  per culture dish was 0.003  $\mu\text{g}$ .

† The data are expressed as geometric mean anti-DNP PFC responses of three cultures.

the IgG and IgM responses to soluble DNP-KLH. Primed spleen cells cultured with DNP-KLH macrophages as the only source of in vitro immunogen (group C) developed significant secondary responses, of both antibody classes, which were higher than those obtained in control cultures (groups A and B). Note that the ratio of IgM to IgG antibody responses to DNP-KLH macrophages (group C) is essentially reversed from the ratio obtained to soluble DNP-KLH in the control cultures (group A) and in cultures containing normal macrophages (group B).

The specificity of macrophage-bound DNP-carrier conjugates was tested in the secondary in vitro anti-DNP response system as follows. Spleen cells from A/St mice primed with either DNP-KLH or DNP-BGG were cultured separately with  $5 \times 10^5$  DNP-KLH macrophages and  $5 \times 10^5$  DNP-BGG macrophages. Control cultures consisted of each primed cell population incubated without any in vitro antigen. The results are illustrated in Fig. 2. The DNP-

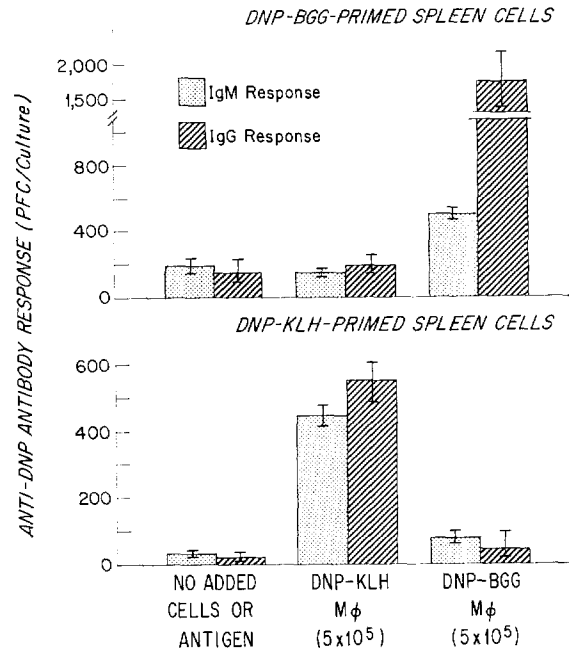


FIG. 2. Specificity of macrophage-bound DNP-proteins in in vitro secondary anti-DNP antibody responses. Spleen cells from A/St mice primed with either DNP-BGG (2 mo earlier, top panel) or DNP-KLH (3 mo earlier, bottom panel) were cultured separately with  $5 \times 10^6$  DNP-KLH macrophages (M $\phi$ ) and  $5 \times 10^6$  DNP-BGG M $\phi$ . The quantities of DNP-KLH and DNP-BGG on macrophages were 0.003 and 0.038  $\mu$ g, respectively, per culture. Controls consisted of primed cells cultured in the absence of any in vitro immunogen or added M $\phi$ . The IgM and IgG anti-DNP responses are expressed as geometric means of three cultures. Standard errors are represented by vertical bars. A comparison of responses obtained with DNP-BGG-primed cells (top panel) gave the following results: Comparison of the responses to DNP-BGG M $\phi$  with those elicited by DNP-KLH M $\phi$  yielded  $P$  values of  $0.005 > P > 0.001$  and  $0.001 > P$  for IgM and IgG responses, respectively. A comparison of responses obtained with DNP-KLH-primed cells (bottom panel) gave the following results. Comparison of the responses to DNP-KLH M $\phi$  with those elicited by DNP-BGG M $\phi$  yielded  $P$  values of  $0.001 > P$  for both IgM and IgG responses.

BGG-primed spleen cells (top panel) developed very good anti-DNP secondary responses when cultured with DNP-BGG macrophages, whereas the same cells cultured with DNP-KLH macrophages manifested only background levels of anti-DNP PFC. Similarly, DNP-KLH-primed spleen cells (bottom panel) were stimulated in vitro by macrophages bearing the homologous antigen, DNP-KLH, but not by DNP-BGG macrophages. The fact that the "carrier effect" (22) is maintained when such conjugates are presented on macrophages indicates that antigen presentation by these cells is highly efficient in triggering both T and B lymphocytes.

*Characterization of Cells Capable of Binding and Presenting Antigen to Im-*

*munocompetent Lymphocytes.*—The preceding experiments clearly demonstrate that DNP-carrier conjugates bound to macrophages serve as highly efficient stimuli for secondary anti-DNP antibody responses in vitro. These results may reflect the effectiveness of presentation of antigenic determinants on the background of a surface membrane, but do not exclude a more specialized function for such cells (23, 24). The following series of experiments were designed to analyze more precisely the characteristics of cells capable of handling antigen in this manner. Specifically, we were interested in determining whether fibroblasts could serve as membrane surfaces for effective antigen presentation.

*The uniqueness of macrophages as handlers of antigen: capacity of fibroblasts to bind DNP-carrier conjugates and stimulate secondary in vitro anti-DNP responses:* In a preliminary experiment, cells from DNP-KLH-primed A/J mice were cultured with and without soluble DNP-KLH either alone or in the presence of varying quantities of normal syngeneic fibroblasts. The results of this experiment (not shown) demonstrated that a quantity of  $5 \times 10^4$  fibroblasts per culture had neither deleterious nor enhancing effects on the secondary in vitro anti-DNP response. Higher quantities of fibroblasts were clearly inhibitory. Subsequent experiments were carried out with this number of fibroblasts ( $5 \times 10^4$ /culture).

A typical experiment comparing the capacities of DNP-KLH macrophages and DNP-KLH fibroblasts to stimulate anti-DNP secondary responses in vitro is illustrated in Fig. 3. DNP-KLH-primed spleen cells were cultured with and without soluble DNP-KLH, either alone or together with  $5 \times 10^4$  DNP-KLH macrophages or DNP-KLH fibroblasts. Two points are particularly noteworthy about the data. First, the fibroblasts were as effective as macrophages in presenting immunogenic moieties of DNP-KLH (note again the apparent favoring of IgG responses by cell-bound antigen). Secondly, the IgG responses obtained with macrophage- or fibroblast-bound DNP-KLH were clearly inhibited in such cultures when soluble DNP-KLH was added to the culture. The degree of inhibition was only one to make the response equivalent to those obtained in control cultures containing spleen cells alone with soluble DNP-KLH. However, no such inhibition was observed in the IgM responses.

*Relevance of the cell membrane as a backbone for antigen presentation:* This experiment was designed to determine whether the increased efficiency of antigen presentation on macrophages or fibroblasts vs. soluble antigen was simply a matter of antigen stabilization on a relatively immobile surface. We have approached this question by performing a comparative study of the strength of immunogenic signals conferred on primed spleen cells by soluble antigen, macrophage-bound antigen, and antigen stabilized by covalent coupling to the culture dish itself.

The procedure for coupling DNP-KLH covalently to plastic Petri dishes was as follows. Varying quantities of DNP-KLH, in 0.5-ml vol, were reacted with 35-mm plastic dishes in the presence of 50 mg (in 1.0 ml of Hanks' balanced salt solution) of water-soluble carbodi-

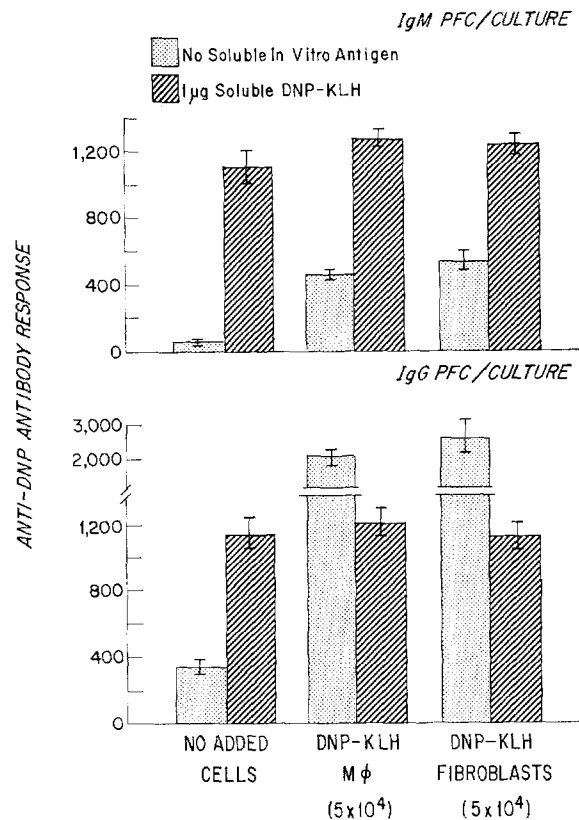


FIG. 3. Capacity of fibroblasts to bind DNP-KLH and stimulate secondary in vitro anti-DNP responses. DNP-KLH-primed spleen cells from A/St donors (primed 3 mo earlier) were cultured with and without soluble DNP-KLH ( $1 \mu\text{g}/\text{culture}$ ), either alone or together with  $5 \times 10^4$  DNP-KLH macrophages (containing  $0.20 \text{ ng DNP-KLH}$ ) or DNP-KLH fibroblasts (containing  $0.07 \text{ ng DNP-KLH}$ ). Control cultures consisted of primed cells cultured in the absence of any added macrophages or fibroblasts. The IgM (top panel) and IgG (bottom panel) responses are expressed as geometric means of three cultures. Vertical bars indicate ranges of standard errors. A comparison of responses elicited by DNP-KLH macrophages or DNP-KLH fibroblasts in cultures containing no soluble antigen to appropriate controls yielded  $P$  values of  $0.001 > P$  for both IgM and IgG responses in each case. Responses obtained in cultures containing  $1 \mu\text{g}$  of soluble DNP-KLH were not statistically different irrespective of whether or not DNP-KLH macrophages or fibroblasts were present.

imide (ECDI) for 1 h at ambient temperature. The dishes were then washed 12 times with 3.0 ml of medium before use. In a preliminary study with DNP- $^{125}\text{I}$ KLH it was possible to quantitate precisely the amount of conjugate covalently bound to each dish.

Spleen cells from DNP-KLH-primed A/J mice were cultured in the presence of (a) soluble DNP-KLH at doses of 0.001, 0.01, 0.1, and  $1.0 \mu\text{g}$  per culture; (b) DNP-KLH covalently bound to dishes in the same doses as in a; and (c)  $5 \times 10^5$  DNP-KLH macrophages containing 0.0005, 0.005, and  $0.05 \mu\text{g DNP-KLH}/\text{culture}$ .



As shown in Fig. 4, minute quantities of macrophage-bound DNP-KLH (0.0005  $\mu\text{g}$ ) elicited quite striking secondary anti-DNP antibody responses, which, in fact, were substantially higher than the maximal response obtained with soluble DNP-KLH. In contrast, DNP-KLH covalently coupled to dishes failed to induce anything more than rather meager responses, even at the highest antigen dose. Control cultures (not shown) ruled out any possible toxic

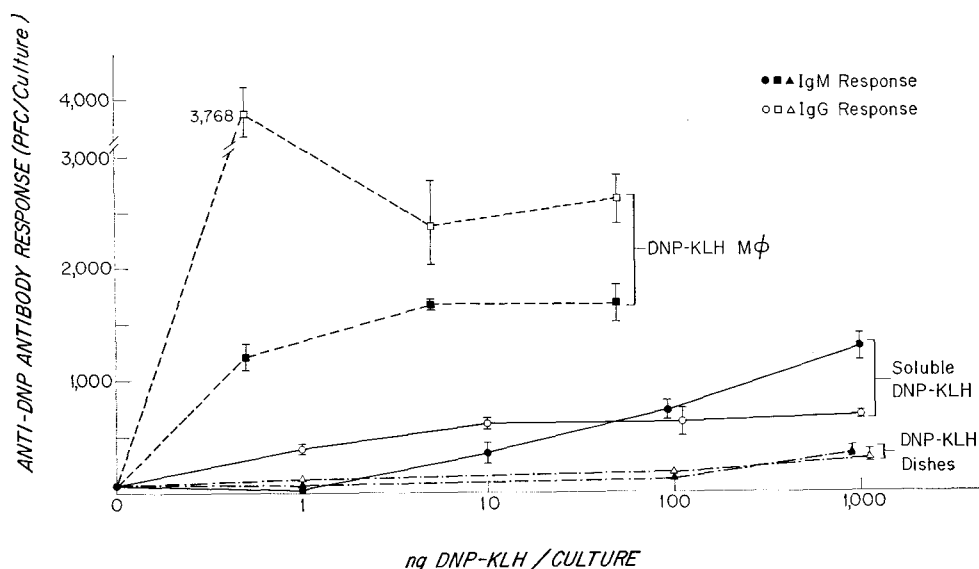


FIG. 4. Relevance of the cell membrane as a backbone for antigen presentation. DNP-KLH-primed spleen cells from A/J donor mice (primed 3 mo earlier) were cultured in the presence of (a)  $5 \times 10^5$  DNP-KLH macrophages containing 0.5, 5.0, and 50.0 ng DNP-KLH/culture; (b) soluble DNP-KLH at doses of 1, 10, 100, and 1,000 ng/culture; and (c) DNP-KLH covalently bound to dishes in the same doses as b. The IgM and IgG responses are expressed as geometric means of three cultures. Vertical bars indicate ranges of standard errors. Control cultures, consisting of primed cells cultured in the absence of any in vitro antigen (not shown), had mean background PFC values of 50 and 20 PFC/culture for IgM and IgG, respectively.

effects of dishes that had been treated with ECDI, since perfectly normal secondary responses to soluble DNP-KLH could be elicited from cells cultured in such dishes.

*Effect of X irradiation on the capacity of macrophages to present antigen.* Previous studies have demonstrated that X irradiation has little or no effect on the immunogenicity of most antigens when bound to macrophages (25, 26). We have tested this in the present study by comparing the capacity of nonirradiated or irradiated DNP-KLH macrophages to elicit secondary anti-DNP responses in vitro, using spleen cells from DNP-KLH-primed A/J mice. As shown in

Fig. 5, comparable secondary IgG anti-DNP antibody responses were obtained with both nonirradiated and X-irradiated DNP-KLH macrophages.

*Failure of preincubation with anti-immunoglobulin antibody to inhibit antigen uptake by macrophages and fibroblasts:* The capacity of macrophages to bind antigen directly in the absence of any contributing antibody has been documented in earlier investigations (27, 28). The possible contribution to antigen

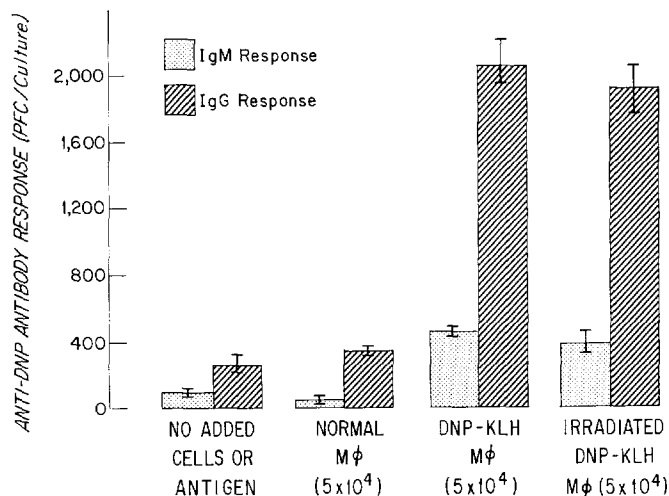


FIG. 5. Effect of X irradiation on the capacity of macrophages to present antigen. Spleen cells from A/J mice primed 2 mo earlier with DNP-KLH were cultured either alone, with  $5 \times 10^4$  normal macrophages (Mφ) (not incubated with DNP-KLH), or with DNP-KLH Mφ from nonirradiated donor mice or from donor mice irradiated (660 R) 24 h before sacrifice. The quantities of DNP-KLH on macrophages were around  $0.003 \mu\text{g}/\text{culture}$ . The IgM and IgG anti-DNP PFC responses are expressed as geometric means of three cultures. Vertical bars indicate ranges of standard errors. A comparison of the IgM and IgG responses elicited by either nonirradiated or irradiated DNP-KLH Mφ with background PFC in control cultures (either containing no added cells or  $5 \times 10^4$  normal Mφ) yielded  $P$  values of  $0.001 > P$  in all cases. Comparison of the responses elicited by nonirradiated DNP-KLH Mφ with those elicited by irradiated DNP-KLH Mφ yield  $P$  values of  $0.05 > P > 0.90$ .

uptake of natural antibodies adsorbed to the surface membrane of either macrophages or fibroblasts in the present studies was examined as follows.

Macrophages or fibroblasts were incubated with RAMG for 30 min on ice ( $120 \mu\text{g}$  of RAMG/ $5 \times 10^4$  cells). After thorough washing, a portion of these cells and others not exposed to RAMG were reacted with DNP-KLH ( $1 \mu\text{g}/5 \times 10^4$ ). Quantitative uptake of DNP-KLH by macrophages or fibroblasts was not affected by RAMG pretreatment. The relative capacity of the various macrophage and fibroblast preparations to stimulate secondary in vitro anti-DNP responses in A/J DNP-KLH-primed spleen cells was evaluated in the usual manner.

As shown in Fig. 6, RAMG-treated DNP-KLH macrophages were as effective as untreated DNP-KLH macrophages in stimulating significant IgG secondary

responses. Note again the partial inhibition in cultures containing soluble as well as cell-bound DNP-KLH. Control cultures containing normal macrophages or RAMG-treated macrophages not exposed to DNP-KLH did not differ significantly in their responses to soluble DNP-KLH from those manifested by spleen cells alone. Precisely the same results (not shown) were obtained with

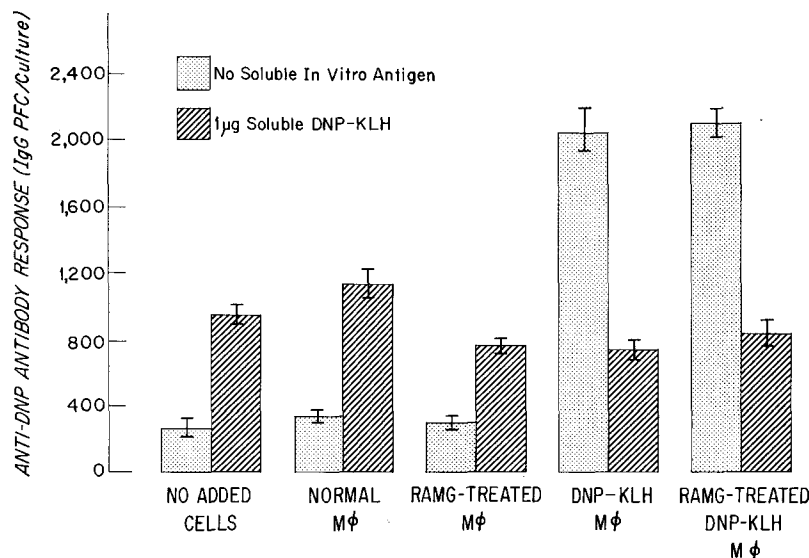


FIG. 6. Failure of RAMG preincubation to inhibit antigen uptake by macrophages. DNP-KLH-primed spleen cells from A/J donors (primed 3 mo earlier) were cultured with and without soluble DNP-KLH ( $1 \mu\text{g}/\text{culture}$ ), either alone or together with either  $5 \times 10^4$  (a) normal macrophages (Mφ); (b) RAMG-treated Mφ ( $120 \mu\text{g}$  RAMG/ $5 \times 10^4$  cells, 30 min on ice, then washed); (c) DNP-KLH Mφ; or (d) RAMG-pretreated DNP-KLH Mφ. The quantity of DNP-KLH on untreated and RAMG-pretreated Mφ was  $0.0031 \mu\text{g}/\text{culture}$ . The secondary IgG anti-DNP PFC responses are expressed as geometric means of three cultures. Vertical bars indicate ranges of standard errors. A comparison of responses elicited by DNP-KLH Mφ or RAMG-treated DNP-KLH Mφ in cultures containing no soluble antigen to the three control groups yielded  $P$  values of  $0.001 > P$  in all cases. Responses obtained in cultures containing  $1 \mu\text{g}$  of soluble DNP-KLH were not statistically significant by comparison, irrespective of the types of additional cells present in the culture.

fibroblasts. Hence, these experiments definitively exclude a possible contribution of antibody to the uptake of antigen by macrophages or fibroblasts and demonstrate that such cells can indeed bind antigen directly in a highly immunogenic form.

*Relative efficiency of antigen bound directly or via immune complexes to macrophages and fibroblasts:* The existence of a specific receptor on the macrophage surface membrane for a site on the Fc portion of the Ig molecule provides another means by which antigen can become attached to such cells (29, 30). There are ample data in the literature supporting the point that antigen uptake

by macrophages is quantitatively greater when it is in the form of an immune complex (31). There are no hard data, however, to indicate whether or not antigen on macrophages is more immunogenic in the form of immune complexes than an equimolar quantity of the same antigen bound directly to the membrane. In contrast to macrophages, there is no Fc receptor known to exist on fibroblasts. Indeed, our line of fibroblasts did not bind SRBC coated with anti-SRBC antibody as the macrophages did. This provides a point of distinction between antigen presentation by the two cell types in that at limiting antigen doses it should be possible to significantly increase immunogenicity of antigen bound via immune complexes to the macrophage membrane while failing to do so in the case of fibroblasts. The following experiments were designed to ascertain this point.

Spleen cells from DNP-KLH-primed A/J mice were cultured in the presence of either macrophages or fibroblasts that had been incubated with immune complexes prepared with log increments of DNP-KLH and high-titered (1.5 mg/ml) mouse anti-DNP antibody in a 1:1  $\mu\text{g}/\mu\text{l}$  ratio. (Preliminary studies had shown this ratio to cause a fourfold increase in antigen uptake by macrophages.) Thus immune complexes prepared with 0.01, 0.1, and 1.0  $\mu\text{g}$  of DNP-KLH were employed. As controls, macrophages and fibroblasts were incubated with the same quantities of DNP-KLH in the presence of an antihuman serum albumin (HSA) antiserum (also at a 1:1 ratio).

The degree of antigen uptake on macrophages and fibroblasts in the presence of specific and nonspecific antibodies is listed in the legend to Fig. 7, which illustrates the results. No major differences in the IgM anti-DNP PFC responses to the various cell-bound antigens were found (left panel). The significant data were obtained in the IgG responses (right panel). Although the number of IgG PFC differed very little between cells cultured with macrophages or with fibroblasts incubated with low doses (0.01 and 0.1  $\mu\text{g}$ ) of DNP-KLH in the presence or absence of specific antibodies, a sharp distinction occurred at the highest antigen dose. Thus macrophages incubated with 1  $\mu\text{g}$  of DNP-KLH plus specific anti-DNP antibody elicited significantly higher anti-DNP IgG responses than occurred with macrophages incubated with the same dose of DNP-KLH plus anti-HSA. Moreover, incubation of fibroblasts with DNP-KLH in the presence of anti-DNP antibody created no detectable advantage over anti-HSA with respect to the magnitude of the *in vitro* response obtained.

*Relative capacities of syngeneic and allogeneic antigen-bearing macrophages to stimulate in vitro secondary anti-DNP antibody responses:* Studies performed with antigen-bearing macrophages in *in vivo* systems have shown that across a major histocompatibility barrier, allogeneic macrophages failed to induce immune responses to the antigens they bear (32). On the other hand, where the differences in histocompatibility specificities are minor, positive results have been obtained (33). Such observations are quite consistent with what should be expected in circumstances in which active host rejection reactions against the histoincompatible macrophages play an important role. The question remains

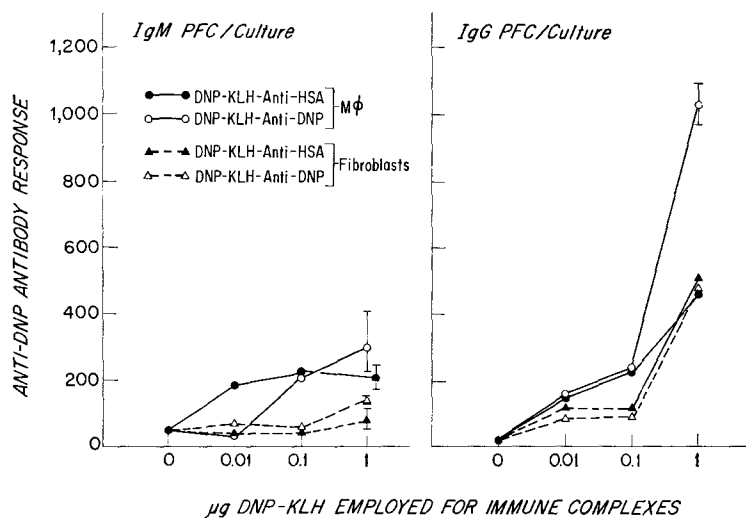


FIG. 7. Relative efficiency of antigen bound directly or via immune complexes to macrophages and fibroblasts. Spleen cells from A/J mice primed 2½ mo earlier with DNP-KLH were cultured in the presence of either macrophages or fibroblasts that had been incubated with immune complexes prepared as described in the text. The degree of antigen uptake (expressed as quantity DNP-KLH per culture) on macrophages and fibroblasts in the presence of specific and nonspecific (anti-HSA) antibodies was as follows (from lowest to highest amounts employed): Macrophages, 0.12, 1.2, and 11.2 ng for anti-DNP; 0.05, 0.5, and 5.0 ng for anti-HSA. Fibroblasts, 0.016, 0.17, and 1.7 ng for anti-DNP; 0.008, 0.08, and 0.8 ng for anti-HSA. The IgM (left panel) and IgG (right panel) anti-DNP PFC responses are expressed as geometric means of three cultures. Vertical bars indicate ranges of standard errors. A comparison of the IgG responses elicited by macrophages bearing DNP-KLH anti-DNP immune complexes prepared at the 1 µg dose with those elicited by immune complex-bearing fibroblasts yielded a  $P$  value of  $0.02 > P > 0.01$ .

open, therefore, as to whether or not macrophages can effectively present antigen to trigger allogeneic lymphocytes. This question is of critical importance in view of the fact that physiologic interaction between T and B lymphocytes in the development of humoral immune responses to antigen occurs rather poorly across major histocompatibility barriers.<sup>2</sup> A possible interpretation of the latter point is that perhaps antigen presentation to T and B lymphocytes is less effective on allogeneic macrophages. We put this possibility to the test in the following experiment.

Spleen cells from DNP-KLH-primed A/St and BALB/c mice were cultured in the presence of soluble DNP-KLH, or  $5 \times 10^5$  DNP-KLH-bearing macrophages. Primed cells of each

<sup>2</sup> Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histocompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral responses to hapten-protein conjugates. Manuscript submitted for publication.

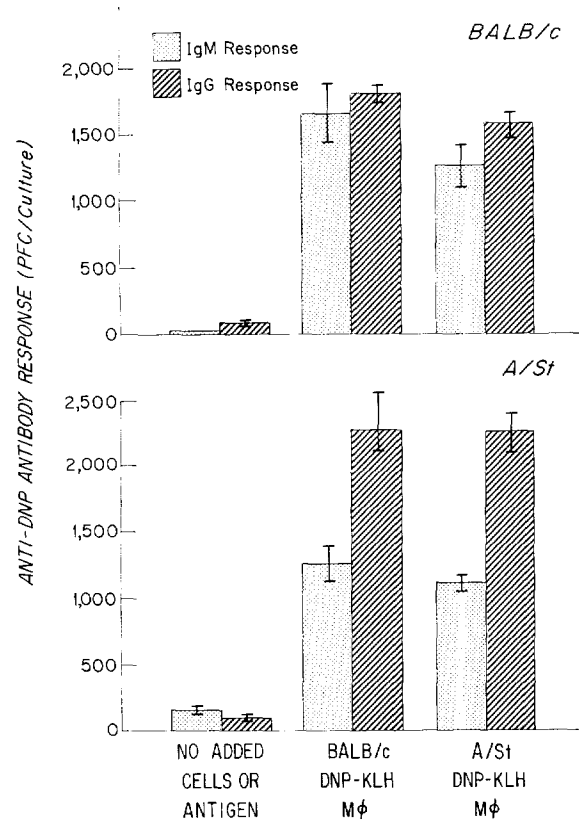


FIG. 8. Relative capacities of syngeneic and allogeneic antigen-bearing macrophages to stimulate in vitro secondary anti-DNP responses. Spleen cells from DNP-KLH-primed A/St (lower panel) and BALB/c (upper panel) mice were cultured in the presence of soluble DNP-KLH, or  $5 \times 10^5$  DNP-KLH macrophages ( $M\phi$ ). Primed cells of each strain were exposed, in separate cultures, to syngeneic and allogeneic DNP-KLH  $M\phi$  containing 0.003–0.004  $\mu\text{g}$  of DNP-KLH/culture. Control cultures consisted of primed cells cultured in the absence of any added cells or in vitro antigen. The IgM and IgG anti-DNP PFC responses are expressed as geometric means of three cultures. Vertical bars represent ranges of the standard errors. A comparison of IgM and IgG responses elicited by either syngeneic or allogeneic DNP-KLH  $M\phi$  with respective control cultures yielded  $P$  values of  $0.001 > P$  in all cases. No statistically significant difference existed between responses (of either antibody class) elicited by syngeneic vs. allogeneic DNP-KLH  $M\phi$ .

strain were exposed, in separate cultures, to syngeneic and allogeneic DNP-KLH macrophages and assayed for IgM and IgG anti-DNP PFC 4 days later.

As shown in Fig. 8, primed spleen cells from both strains developed highly significant secondary responses to macrophage-bound DNP-KLH irrespective of whether the macrophages were obtained from syngeneic or allogeneic donors. This result makes it highly improbable that the macrophage can be blamed for any defect in physiologic T-B cell interaction across histocompatibility barriers.

*The Requirement for Macrophages in Secondary In Vitro Anti-DNP Antibody Responses.*—In the following experiments we have examined the requirement for macrophages in our own culture system of secondary responses to DNP-KLH.

Macrophages were depleted by the technique described by Mosier (34). This procedure was repeated three to four times, and the final suspension of nonadherent (NA) spleen cells was washed and then examined cytologically for the presence and quantity of macrophages and immunohistochemically for the number of lymphocytes with surface Ig. The initial suspension of primed spleen cells contained 5–6% typical macrophages and about 45% of lymphocytes with surface Ig, i.e., B lymphocytes. Adherence to dishes substantially depleted the cells of all typical macrophages. However, the suspension still contained about one to two cells per 1,000 that morphologically exhibited certain monocytoïd features and may or may not have been macrophages. The number of B lymphocytes was depleted 20–25% after three or four cycles of adherence to dishes. The NA cells were then placed in culture either alone or with the addition of  $5 \times 10^5$  normal macrophages or DNP-KLH macrophages and were tested for their capacity to develop secondary anti-DNP PFC to soluble DNP-KLH. A control group consisting of NA spleen cells preincubated with  $1 \mu\text{g}$  DNP-KLH/ $12 \times 10^6$  cells for 30 min on ice and then thoroughly washed before culture was included in one experiment.

Certain basic points emerge from two such experiments shown in Fig. 9: (a) primed spleen cells extensively depleted of adherent cells nevertheless developed substantial secondary IgM and IgG in vitro responses to soluble DNP-KLH; (b) the addition of normal macrophages to cultures of NA cells improved the response to soluble DNP-KLH, though not dramatically; (c) a substantial secondary anti-DNP response was obtained with primed NA cells that had been only briefly exposed to DNP-KLH in the cold before culture; and (d) the presentation of DNP-KLH in small quantities on macrophages elicited the most significant and consistent secondary anti-DNP responses, particularly of the IgG class, and this was clearly an antigen dose-related effect.

#### DISCUSSION

The present studies have demonstrated the capacity of macrophages to bind and retain antigen in immunogenic form in in vitro secondary antibody responses to DNP-protein conjugates. These results indicate, moreover, that the required interaction of T and B lymphocytes initially demonstrated by others (35–37) occurs with antigenic determinants bound to the macrophages. As reported previously by others (2), the capacity of macrophages to bind and present antigen was not affected either by previous X irradiation of the cell or by preincubation with antimouse immunoglobulin antibodies. The latter finding rules out any contribution to antigen uptake of natural antibodies adsorbed to the cell surface membrane. The present studies emphasize the fact that despite uptake of antigen by macrophages, cells that degrade antigen by virtue of their content of hydrolytic enzymes, the native determinants of the molecules, have been maintained. This conclusion is particularly valid on the basis of results from the present experiments utilizing DNP-protein conjugates. An explanation for the apparent discrepancy may be that not all of the antigen is degraded by

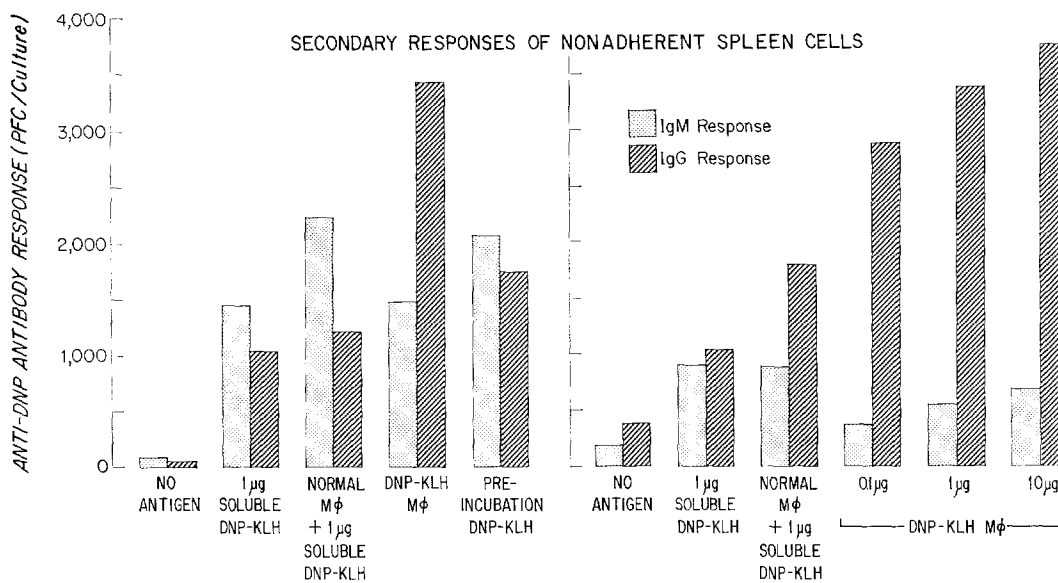


Fig. 9. Secondary in vitro anti-DNP antibody responses in cultures of primed nonadherent spleen cells. DNP-KLH-primed A/St spleen cells were depleted of adherent cells (see text) and then tested for their capacity to develop secondary anti-DNP responses in vitro. Two experiments are shown. In the left panel, cells were cultured alone or with the addition of  $5 \times 10^5$  normal M $\phi$  and tested for responses to  $1 \mu\text{g}$  of soluble DNP-KLH. Another culture group consisted of nonadherent cells exposed to  $5 \times 10^5$  DNP-KLH M $\phi$  (as the only source of antigen), and the last group consisted of nonadherent cells that had been preincubated with DNP-KLH for 30 min on ice and then washed before culture. In the right panel, nonadherent cells were cultured under conditions similar to those in the left panel with the exception that three different doses of DNP-KLH were used for macrophage preincubation. In the latter case, the quantities of DNP-KLH bound to macrophages per culture were 0.00025, 0.0025, and 0.025  $\mu\text{g}$ , respectively. The mean secondary IgM and IgG anti-DNP responses are illustrated.

macrophages, those few molecules that escape degradation being the molecules that remain on the cell membrane surface for a finite period of time (2).

We have also shown here that antigen presentation is not a property unique to macrophages, in that fibroblasts possess a comparable capacity for binding and effectively presenting antigen to trigger immunocompetent lymphocytes, a finding not heretofore reported. The capacity of fibroblast-bound antigen to elicit immune responses was actually first observed in *in vivo* transfer experiments during the course of unpublished studies by André Cruchaud and one of us (E. R. U.). Although earlier attempts to transfer immunogenic moieties with cell types other than macrophages were unsuccessful (38), there appear to be reasonable explanations for these failures. First, such studies employed *in vivo* systems in which the different patterns of migration of various cell types may complicate interpretation of results. Secondly, Mitchison's failure to elicit in



vivo responses with antigen-bearing fibroblasts (32) may be explained by the fact that the cells were trypsinized after antigen uptake, a procedure that certainly must have removed cell surface-bound immunogen. In contrast, in the present study and in those of Cruchaud and Unanue, the fibroblasts were trypsinized before exposure to the antigen.

Nonetheless, a clear point of distinction with respect to antigen uptake by these two cell types exists by virtue of the presence, on surface membranes of macrophages but not fibroblasts, of specific receptors for Ig. Thus, at limiting antigen doses it was possible to increase appreciably the immunogenicity of DNP-KLH bound in the form of immune complexes to the macrophage membrane, whereas this was not the case with fibroblasts. It would appear, therefore, that the uniqueness of macrophages insofar as the immune system is concerned is related primarily to: (a) their geographic distribution in lymphoid organs in proximity to immunocompetent lymphocytes, (b) the existence of specific receptors for Ig and the third complement component, and (c) perhaps their capacity to respond to products of lymphocyte-antigen interaction. Certainly, this cell does not appear to play a unique role with respect to a processing step for handling antigen.

On the other hand, presentation of antigenic determinants bound to cell surface membranes, be it on macrophages or fibroblasts, exhibits an exquisite degree of sensitivity in terms of the triggering threshold of specific lymphocytes. This conclusion derives from several observations. First, the effective antigen dose for induction of optimal secondary *in vitro* anti-DNP responses was consistently several logs lower for cell-bound DNP-KLH than for soluble DNP-KLH. More pertinent, perhaps, is the observation that this was particularly true for antibody responses of the IgG class. Secondly, there was a consistent inhibitory effect of soluble DNP-KLH on the anti-DNP response to cell-bound DNP-KLH. By way of an explanation for this, an important point is that the observed inhibition was highly selective in that IgM antibody responses were completely unaffected whereas IgG production was diminished. However, the IgG antibody response was still comparable with control cultures stimulated with soluble DNP-KLH but not containing cell-bound antigen. These results are difficult to explain on the basis of a possible feedback inhibitory influence by IgM antibodies since there were essentially no differences in the IgM responses between control cultures (containing soluble DNP-KLH alone) and cultures containing both soluble and cell-bound DNP-KLH. A valid explanation may be that certain cells are preferentially (perhaps necessarily) triggered by cell-bound, rather than soluble, antigen and that these cells, upon directly binding antigen molecules in the soluble state, are either transiently inhibited (by virtue of receptor blocking) from being triggered, or even, perhaps, rendered tolerant. The possibility that such effects could occur with either T or B lymphocytes (or both) is further discussed below.

Another point to be made concerning cell-bound antigen and lymphocyte

triggering is the importance of the cell membrane itself. The experiment comparing the inductive capacity of cell-bound DNP-KLH to DNP-KLH covalently bound to the culture dish implies that enhanced triggering by cell-bound antigen is not merely a result of the existence of determinants on a relatively immobile surface. Alternatively, the failure with dish-bound antigen may reflect the requirement for readily accessible determinants, perhaps for endocytosis, in order for effective lymphocyte triggering to occur. The contribution of the macrophage (or fibroblast) cell surface membrane may, therefore, be crucial with respect to determinant concentration and/or spatial orientation upon contact with receptors on specific lymphocytes. Delineation of this point will quite likely be critical to our understanding of signal transmission in immunocompetent lymphocytes.

The final observation relevant to the relationship between cell-bound antigen and sensitivity of lymphocyte triggering derives from the experiments with nonadherent spleen cells. Clearly, primed nonadherent spleen cells developed good secondary *in vitro* anti-DNP antibody responses in our hands. Although there is general agreement concerning the requirement for adherent cells in primary *in vitro* antibody responses (14, 34, 39), the literature is rather contradictory with respect to the secondary *in vitro* response (14, 40-42). The latter situation, still unresolved, may reflect either differences in the cell culture systems (13, 43) or the nature of the antigens employed. Our own results, presented here, could indicate that despite our efforts to maximally deplete such spleen cells of macrophages, there were sufficient numbers remaining to account for the positive secondary responses. We favor an alternative explanation by which primed B lymphocytes in the nonadherent population bind antigen and perform the role of presenting cell-bound determinants to initiate T cell activation, which in turn facilitates triggering of B lymphocytes. This reasoning is supported to some extent by the results with nonadherent cells transiently preincubated with DNP-KLH before culture.

A fundamental question to be considered here is where the macrophage fits in the physiologic sequence of T and B cell interaction. A provocative hypothetical model to explain the nature of this interaction has been very recently proposed by Feldmann and colleagues (44-46) on the basis of some very elegant *in vitro* experiments performed by them. Briefly summarized, their hypothetical scheme pictures antigen-activated T cells as releasing their specific IgT receptors (presumably monomeric IgM) in the form of an immune complex with antigen. This complex attaches to the macrophage surface forming a matrix of determinants capable of triggering specific B lymphocytes (46). Careful examination of this hypothesis in light of our own *in vitro* data raises several serious questions.

(a) Can we safely presume that T cell activation can be triggered by antigen not presented on a macrophage surface? Feldmann's hypothesis implies that this is the case, and, moreover, makes no distinction between unprimed and

primed cell populations, assuming the situation is essentially the same in both cases (46). The potential error in this concept is indicated by the well-established fact that T cell tolerance is rapidly induced in unprimed animals by deaggregated proteins that are poorly taken up by macrophages (47). In contrast, to our knowledge it has not been shown that T cells can be similarly rendered tolerant in a primed animal. An alternative consideration to explain Feldmann's hypothesis is that T cell activation may occur with doses or forms of macrophage-bound antigen that are distinctly different from those required for B cells. However, data in the present studies argue against such a distinction since both T and B cells were triggered by antigen bound directly to macrophage surfaces.

(b) Is it necessary for antigen to be bound to macrophages in the form of immune complexes in order for it to be optimally immunogenic? This question is central because it speaks directly to the necessity for an antigen-specific IgT molecule to be released at all. One of us, with Benacerraf, recently presented arguments, based on hard data, that T lymphocytes exert a sophisticated regulatory influence on B cell responses to antigen (1, 48). This regulatory influence is envisaged to be mediated through the action of activated T cells and/or nonspecific products released by such cells upon suitable triggering (1, 48). The attractiveness of this concept derives, in part, from the fact that it distinguishes the T cell role clearly from that of the macrophage. Feldmann's scheme (46) places the T cell function once again in a position accessory to the role of macrophages, which as we have previously stated may be an unnecessarily wasteful duplication of effort (1).

The latter reasoning may be incorrect if it can be proven that macrophage-bound antigen in the form of an immune complex possesses any unique advantage in triggering lymphocytes. As shown here, macrophages bearing limiting antigen doses in the form of immune complexes induced somewhat higher IgG responses than cells bearing noncomplexed antigen. However, the higher quantitative uptake of complexed antigen by macrophages could account for these differences in secondary responses. Since it is likewise clearly established by these studies that noncomplexed cell surface-bound antigens can elicit quite striking secondary anti-DNP responses, there appears to be no unique advantage in terms of determinant presentation to complexed vs. noncomplexed antigen on macrophage surfaces. Moreover, attempts by us to override the carrier effect by placing antigen on macrophages in the form of immune complexes have been uniformly unsuccessful. In other words, DNP-KLH-primed lymphocytes do not develop secondary anti-DNP responses when cultured with macrophage cell surface-bound DNP-BGG:anti-BGG immune complexes (Katz, D. H., and E. R. Unanue, unpublished observations).

(c) Finally, if the active T cell product has specificity for antigen and serves only to bind antigen to macrophages, why is physiologic T-B cell interaction relatively difficult to demonstrate across major histocompatibility barriers?<sup>2</sup> If the difficulty across the histocompatibility barrier is considered in terms of

Feldmann's hypothesis, then the block must relate to the interaction between antigen-bearing macrophage and the responding allogeneic B cell. We have documented in this study, however, that antigen-bearing macrophages can effectively trigger primed lymphocytes even across the major histocompatibility barrier, suggesting that the defect in physiologic cooperation in allogeneic systems may relate to the actual interaction between T and B cells, a possibility that cannot be explained by IgT-antigen complexes.

Most importantly, the experiments presented here establish that thymus-dependent hapten-protein conjugates that bind directly to macrophage surface membranes are extremely efficient in triggering both carrier-specific T and hapten-specific B lymphocytes. These findings are not compatible with the Feldmann hypothesis, since even in conditions in which the only antigen presented to cells in our cultures was macrophage-bound, the requirement for T cells was not obviated. The argument that the antigen might come off macrophages, attach to T cells, and then be presented as the postulated complex when bound to macrophages is not tenable when one considers that directly bound macrophage-antigen was several logs more efficient than free antigen in stimulating antibody production in our cultures.

In summary, we envision the following sequence of events after the entrance of antigen into lymphoid tissue of an unimmunized individual: (*a*) antigen is concentrated by macrophages and other accessory-type cells, (*b*) antigen-committed lymphocytes of the recirculating pool are trapped along the sites of antigen concentration (49, 50), (*c*) T lymphocytes are triggered by the cell-bound antigen and become activated, and (*d*) the activated T cell and/or its nonspecific mediators regulate the triggering of nearby B lymphocytes at some time during or after the interaction of these B lymphocytes with antigen. These events are modified in an immune animal by the presence of antibodies that favor the concentration of antigen on macrophages or B lymphocytes, by a larger number of primed B cells (which could serve as an antigen-concentrating cell), and by primed T lymphocytes having different threshold levels of activation.

#### SUMMARY

A detailed analysis of the role of determinant presentation in the process of triggering immunocompetent lymphocytes has been made utilizing cell-bound hapten-carrier conjugates to elicit secondary antihapten antibody responses, primarily *in vitro*. The results of these experiments demonstrate that: (*a*) hapten-protein conjugates will attach to the surface membranes of macrophages directly, in the absence of specific antibodies, in a highly immunogenic form; (*b*) such macrophage-bound conjugates serve as remarkably efficient stimuli to trigger both thymus-derived (T) and bone marrow-derived (B) cells in a specific manner, lowering the optimal threshold antigen dose (in molar terms) by several logs as compared with soluble antigen; (*c*) the macrophage is not unique in this regard, since fibroblasts are essentially comparable in the capacity to

present antigen in highly immunogenic form; (*d*) cell surface-bound antigen clearly favors secondary in vitro responses of the IgG as compared with the IgM antibody class; (*e*) in terms of triggering B or T cells, antigen bound to macrophages in the form of immune complexes does not appear to possess any appreciable advantage over equimolar quantities of directly attached antigen; (*f*) the increased immunogenicity of cell-bound antigen appears to reflect certain crucial, and undefined, features of cell surface membranes and not merely the stabilization of determinants on a relatively immobile surface; and (*g*) although the efficiency of lymphocyte triggering is markedly enhanced by cell-bound antigen, the presence of macrophages is apparently not an absolute requirement for eliciting secondary in vitro antibody responses to soluble hapten-protein conjugates.

The relevance of these observations to the nature of the signal induced upon antigen interaction by specific lymphocytes and the sequential cellular events involved in the regulatory influence of activated T cells on B cell responses to antigen is discussed. We postulate that T lymphocytes are best triggered by cell-bound antigen and that after this step the activated T lymphocytes regulate the triggering of B cells with antigen.

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