THE PROLIFERATIVE AND ANAMNESTIC ANTIBODY RESPONSE OF RABBIT LYMPHOID CELLS IN VITRO

II. Requirement for Adherent and Nonadherent Cells of the Responses to Particulate Antigens in Spleen Cell Cultures*

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Primary and secondary immune responses to sheep erythrocytes have been obtained in tissue cultures of dispersed mouse spleen (1, 2), and such cultures provide a means of investigating, at the cellular level, the in vitro antibody response. The results of Mosier (3) suggest that at least two different cell populations in mouse spleen suspensions are required for the primary response to sheep erythrocytes in vitro, one with propensity for adhering to glass or plastic surfaces, and the other a nonadherent population. It has been suggested that the "adherent" cells, which have been shown to be non-thymus-dependent (4), insensitive to tolerance induction (5), and resistant to 1000 R X-irradiation (6) are primarily macrophages. These cells can be incubated with the erythrocyte antigen, and then after washing recombined with the nonadherent cell population for production of a primary response. The role of these adherent cells in the secondary response is not established, since it has been reported recently that nonadherent spleen cells from sensitized mice may give a secondary immune response to sheep erythrocytes in vitro (7).

The present studies were undertaken to examine whether primary and secondary responses could be induced in cultures of dispersed rabbit spleen cells, and to determine whether adherent and nonadherent cells were also required for an immune response in the rabbit spleen cell culture system. Since it was found that an antibody response could be obtained to *Brucella abortus* antigen as well as to sheep erythrocytes in rabbit spleen cell cultures, it was possible to extend the study of in vitro cell interactions to the immune response to a bacterial antigen.

The results shown below indicate that the in vitro immune responses of nor-

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mal and primed rabbit spleen cells to either *B. abortus* or to sheep erythrocytes are dependent on the presence of both adherent and nonadherent cell populations. However, the requirement for adherent cells was more readily demonstrated in dish than in tube cultures, since separation of nonadherent from adherent cells more consistently resulted in suppression of the immune responses in the dish cultures. Even in experiments where removal of the adherent cells resulted in a depression of the secondary response in tube cultures, a parallel depletion of the proliferative response to the same antigen was not obtained.

Materials and Methods

Animals.—Normal New Zealand rabbits of 1–2 kg were used as tissue donors for cell cultures in experiments on the primary response. Similar rabbits, sensitized by intravenous injection of 0.5 ml of a 20% suspension of sheep erythrocytes (SE)[1] and 0.5 ml of a 1:2 dilution of a stained ring test antigen of *B. abortus* (BA) (kindly provided by Dr. C. E. Watson from the U. S. Department of Agriculture), were used as tissue donors at various times after sensitization for the studies on the secondary response. Tissues from normal LAF_1 mice (Jackson Memorial Laboratories, Bar Harbor, Maine) were used, or tissues from LAF_1 mice sensitized by intravenous injection of 0.1 ml of a 2% or 20% SE suspension. Some LAF_1 mice were also primed by intravenous injection of 0.1 ml of BA antigen diluted 1:6.

Antigens for Tissue Culture.—The SE and BA antigens used in the cell cultures were prepared by the following procedures. Sterile SE were washed three times in sterile saline and resuspended to 2% in Hanks' balanced salt solution (Hanks' BSS). Sterile BA antigen was prepared by washing the killed organisms several times in saline and resuspending them in saline to the original volume. This suspension was then autoclaved and diluted 1:500 (approximately 2.5×10^7 organisms/ml) in sterile Hanks' BSS for use in cultures.

Preparation of Dispersed Cell Cultures.—Cell suspensions of both mouse spleen and rabbit spleen were prepared by teasing the tissues in Hanks' BSS. Dish cultures were prepared following the procedure outlined by Mishell and Dutton (1, 2). However, the culture media were modified for both rabbit and mouse spleen cultures. Mouse spleen cells were cultured in dishes (35 × 10 mm) at concentrations of $1–2 \times 10^7$ cells per dish in Medium 199 supplemented with 10% fetal calf serum (Lot No. 156, Reheis Chemical Co., Chicago, Ill.). The dishes were rocked and kept in an atmosphere of 7% O_2, 10% CO_2, 83% nitrogen. These dish cultures received daily additions of 0.1 ml nutrient cocktail containing 25% fetal calf serum (1). Rabbit spleen cells at concentrations of $1–2 \times 10^7$ were cultured in a modified Eagle's medium, (8) supplemented with 20% normal rabbit serum, in rocking dishes, under conditions similar to the mouse spleen cultures. Dish cultures were given daily supplements of 0.05 ml nutrient cocktail without fetal calf serum.

Tube cultures at concentrations of $2–4 \times 10^6$ cells of either rabbit or mouse spleen suspensions were prepared in the same media as used in the dish cultures. However, the tubes (12 × 75 mm) were maintained stationary and upright in an atmosphere of 5% CO_2 in balanced air, and the tube cultures were not supplemented with nutrient cocktail during the culture period.

Antigens were added to cultures in portions of 0.05 ml at the time of culture preparation. Both BA and SE antigens were added simultaneously, since it was shown that the in vitro antibody response to one antigen did not interfere with that to the other.

Separation of Adherent and Nonadherent Cells.—Functionally distinct cells from spleen cell

[1] *Abbreviations used in this paper:* BA, *Brucella abortus*; BSS, balanced salt solution; PFC, plaque-forming cells; PHA, phytohemagglutinin; SE, sheep erythrocytes.

suspensions may be separated by virtue of their tendency to adhere or not adhere to glass surfaces (3). In the present experiments, removal of adherent cells was effectively accomplished by incubating suspensions of spleen cells, at a concentration of 10^8 cells/ml in culture medium, on a cotton wool column packed in plastic 12 ml syringes. After incubation for 60 min at 37°C, the nonadherent cells were expressed from the cotton and were used for cultures. It was found that cell suspensions prepared from spleens of sensitized rabbits had to be subjected to two or three passages through cotton wool in order to effect a significant depletion of the antibody response.

Measurements of the Immune Responses In Vitro.—For measurement of the proliferative response to antigen (9), tube cultures received 1 μCi/^{3}H-thymidine (0.36 Ci/mmole, Schwarz Bio Research, Orangeburg, N.Y.) usually 24 hr after initiation of cultures. Incorporation of radioactivity into perchloric acid–insoluble material of cells was measured over a 24 hr period.

TABLE I

Primary Immune Response in Vitro in Rabbit Spleen Cells before and after Removal of Macrophages

Primary response to *Brucella abortus*			Primary response to sheep erythrocytes		
Reciprocal agglutinin titers*		Effective depletion	Plaque-forming cells/10^6‡		Depletion‖
Whole spleen	Depleted§ spleen		Whole spleen	Depleted§ spleen	
					%
8	8	−	36	17	50
4	2	+	127	52	60
8	<2	+	18	1	92
4	<2	+	62	5	92
32	<1	+	490	8	98
			96	14	85
			42	2	95
			24	2	90

* Media collected for titration on day 5 of incubation period.

‡ Cells harvested on day 4 of incubation period.

§ To effect depletion rabbit spleen cell suspensions were passaged through syringes packed with absorbent cotton.

‖ Per cent depletion $= \frac{(\text{whole spleen PFC}/10^6 - (\text{depleted spleen PFC}/10^6)}{(\text{whole spleen PFC}/10^6)}$.

The degree of stimulation was calculated as the ratio of counts per minute (cpm) incorporated by cells exposed to antigens over cpm incorporated by control cells. Radioactivity was determined in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

The primary and secondary antibody responses to SE were measured by assaying the cultured cell suspensions, after a 3 to 5-day period in vitro, for the presence of plaque-forming cells (PFC) (10). Culture media were collected for hemagglutinin and for bacterial agglutinin titrations against SE and BA.

RESULTS

The Primary Response.—Assays for PFC performed on day 4 of the culture period showed that rabbit spleen cells in suspension are capable of giving a primary response to sheep erythrocytes in vitro. The culture medium with

20% rabbit serum contained no antigen cross-reactive with SE, such as is found in the fetal calf serum used in medium for murine spleen cell cultures (1). Nevertheless, even in dishes which had not received antigen, there were a few PFC ranging from 0.5 to 4 per 10^6 plated cells, a background slightly higher than that of freshly prepared normal rabbit spleen cells. The average

TABLE II

*Plaque-Forming Cells (PFC) during Secondary Response of Rabbit Spleen Cells in Dish and Tube Cultures**

Depletion treatments‡	Day after primary§	PFC/10^6 in dish cultures			PFC/10^6 in tube cultures		
		Whole spleen	Depleted spleen	Depletion	Whole spleen	Depleted spleen	Depletion
				%			%
I	10	1,900	190	90	5,200	16,200	0
	28	770	145	82	12,500	15,000	0
	40	715	335	52	42,300	33,000	22
II	7	764	13	98	364‖	222‖	39
	13	8,410	75	99	8,400	24,000	0
	17	1,350	65	95	6,000	15,000	0
	20	1,300	215	83	16,350	13,200	19
	28	8,440	4	100	1,000	925	8
	120 + 28	3,400	20	99	10,000	1,625	84
	120 + 42	1,330	7	99	400	58	86
III	11	4,200	550	87	680	233	66
	11	2,440	29	99	530	226	49
	13	2,300‖	315‖	86	1,750‖	1,660‖	5
	19	790‖	4‖	99	570‖	55‖	90

* Dish cultures received 1 to 2 × 10^7 cells, tube cultures contained 4 × 10^6 cells. All cultures were in 1 ml medium.

‡ Depletion of macrophages from spleen cell suspensions was effected by one (I), two (II) and three (III) incubations on absorbent cotton in a syringe.

§ Rabbits were primed intravenously with 0.5 ml of a 20% sheep erythrocyte suspension.

‖ These cultures were assayed after a 3-day culture period; all the other cultures after 4 days.

response in dishes in which cells were cultured in the presence of antigen was 100 per 10^6 plated cells (Table I). Evidently, the culture of dispersed spleen cells in rocking dishes (1) with daily additions of a nutrient cocktail is superior to the method of culturing spleen fragments on the wall of roller tubes (11), since a primary response to sheep erythrocytes was only rarely obtained by the latter method (12). *Brucella abortus* antigen also induced a primary response in a number of cultures. Media harvested on day 5 of the culture period showed *Brucella* agglutinin titers ranging from 1:4 to 1:32 after exposure to antigen,

but no anti-BA agglutinins were detected in control unstimulated cultures (titers < 1).

A single incubation on absorbent cotton resulted in a 50–98% depression of the responsiveness of normal spleen cells to SE (Table I). In those experiments in which the number of PFC was depressed by more than 60%, the ability to respond to *Brucella* was also abolished.

The Secondary Response.—Dispersed spleen cells obtained from rabbits primed to SE and BA responded well to antigen when cultured either in dishes

TABLE III

*Agglutinin Formation to B. Abortus during Secondary Response of Rabbit Spleen Cells in Dish and Tube Cultures**

Depletion treatments‡	Day after primary§	Reciprocal of agglutinating titer in dish cultures			Reciprocal of agglutinating titer in tube culture		
		Whole spleen	Depleted spleen	Depletion‡	Whole spleen	Depleted spleen	Depletion‡
I	7	1024	64	+	256	64	+
	13	1024	256	+	1000	4000	−
	40	128	64	±	128	128	−
II	7	256	64	+	128	256	−
	7	1024	4	+	256	16	+
	7	256	4	+	16‖	1‖	+
	13	1024	2	+	1000	2000	−
	17	1024	64	+	512	128	+
	20	128	32	+	256	512	−
	28	256	2	+	64	128	−

* Dish cultures received 1–2 × 10^7 cells, tube cultures contained 4 × 10^6 cell . All cultures in 1 ml medium.

‡ Depletion of macrophages from spleen cell suspensions was effected by incubation on absorbent cotton in a syringe.

§ Rabbits were primed intravenously with 1.5 × 10^9 killed *B. abortus* organisms.

‖ These cultures were assayed after 3 day-culture period; all the other cultures after 4 days.

or in tubes. The numbers of PFC per 10^6 plated cells on day 4 of the culture period ranged from 715 to 8440 in the dishes and from 364 to 42,000 in the tubes (Table II). With some spleens there was poor correlation between the numbers of PFC obtained in dish and tube cultures. The tube cultures occasionally gave extremely high numbers of PFC when calculated per 10^6 cells. A possible interpretation may be that survival of cells other than those specifically stimulated may have been poorer in tube than in dish cultures. The anti-BA agglutinin titers ranged from 1:128 to 1:1024 in dish cultures and from 1:16 to 1:1024 in tube cultures (Table IV).

One or two incubations on cotton caused a drastic reduction in the respon-

siveness to both antigens when spleen cells were tested in dish cultures, but such treatment had little effect on the secondary responses in tubes. Even though two incubations on cotton resulted in a uniform depression of the response in dishes, the response in tubes was essentially unaffected in a number of experiments. In some experiments, the responses to both antigens in tube cultures were actually increased after the depletion treatment, although they

TABLE IV

*Proliferative Response to Sheep Erythrocytes (SE) of Rabbit Spleen Cells Cultured in Tubes before and after Incubation on Absorbent Cotton**

Day after primary‡	^{3}H-TDR incorporation per 10^6 cells§ before depletion treatment		D.S.‖	^{3}H-TDR incorporation per 10^6 cells§ after depletion treatment		D.S.‖	Depletion in secondary PFC response
	−SE	+SE		−SE	+SE		%
7	690	1170	1.7	690	885	1.3	0
11	500	1270	2.5	485	1785	3.7	55
13	353	1287	3.7	212	868	4.1	0
14	677	4887	7.2	623	3531	5.6	0
19	627	457	0.7	94	182	2.0	90
20	307	1069	3.4	164	460	2.8	19
27	1444	2201	1.5	595	1295	2.2	95
28	279	714	2.5	537	1327	2.4	8
40	575	1195	2.1	153	225	1.5	49
45	204	520	1.8	106	337	3.2	87
120 + 28	915	3810	4.2	108	1812	16.7	84
120 + 42	488	1181	2.4	83	489	5.9	86

* Depletion of macrophages from spleen cell suspensions was effected by incubation on absorbent cotton in a syringe.

‡ Rabbits were primed intravenously with 0.5 ml of a 20% sheep erythrocyte suspension.

§ Sheep erythrocytes, 0.05 ml of 2% suspension, were added at the outset of the culture period. ^{3}H-thymidine, 1 μCi was added after 24 hr, and cultures were harvested at 48 hr.

‖ D.S., degree of stimulation—cpm in cells with antigen/cpm in cells without antigen.

were nearly abolished in dish cultures (Tables II and III). This apparent enrichment of sensitized (memory) cells may have been caused by a preferential removal on the cotton of cells other than those specifically sensitive. In those experiments in which the responsiveness in tubes was ultimately decreased after two or three repeated incubations on cotton, the per cent of the response remaining in tube cultures was invariably higher than in dish cultures.

Incubation of primed mouse spleen cells on cotton resulted in a similarly marked depletion of the ability to give a secondary response in vitro. One passage through cotton was sufficient to reduce the response of primed mouse cells to 0–15% of the whole spleen response, whereas two consecutive incubations on cotton resulted in a virtually complete depression to $<1\%$ of the

PFC obtained with unfractionated cells. Although the mouse spleen cells, taken 6 to 8 days after priming, gave good secondary responses in dish cultures, ranging from 500 to 15,000 PFC per dish on day 4 of the incubation period, tube cultures were not successful. However, more recently it was found that in some experiments a response could be obtained in tube cultures. Under these conditions, a comparison of dish and tube cultures indicated that removal of adherent cells from mouse spleen suspensions was again much less inhibitory for the secondary response in tubes than in dishes. Attempts to initiate a secondary response to BA in dish cultures of mouse spleen cells have been unsuccessful.

The Proliferative Response.—Removal of cotton-adherent cells did not abolish the proliferative response of rabbit spleen cells. For the study of the proliferative response, the cells were maintained in tubes, and the incorporation of ^{3}H-thymidine was usually measured over the 24 to 48-hr interval of the incubation period. In an occasional experiment the incorporation of ^{3}H-thymidine was measured on the 72 to 96-hour period of incubation with similar results. Even though in some of these experiments, the secondary PFC response in tubes was depressed by prior repeated passage of the cells through cotton, the degree of stimulation of the proliferative activity induced by antigen increased, rather than decreased (Table IV). However, as seen in the table, in several experiments, the actual increment in radioactivity incorporated after stimulation by antigen was less in cotton-absorbed cell suspensions than in the untreated suspensions.

DISCUSSION

The experiments presented show that both primary and secondary immune responses may be obtained by exposure of cultures of dispersed rabbit spleen cells to BA and SE. These results confirm previous observations that in dispersed spleen cell cultures of normal and sensitized rabbits and mice antibody to particulate antigens may be formed (1, 2, 13, 14).

In studies of cell interactions for the primary response, mouse spleen cell suspensions have been separated into a macrophage-rich adherent population and a lymphocyte-rich nonadherent population, both of which must be present in cultures to obtain a primary response (3). The present studies indicate that removal of adherent cells from rabbit spleen cell suspensions will also interfere with the primary response to both the SE and the BA antigens. The results further suggest that interaction between two or more cell types in the rabbit and mouse spleen is requisite for the in vitro secondary immune response as well. These findings appear in contradiction to those of Pierce (7), who found that three passages in plastic dishes did not deplete sensitized mouse spleen cell suspensions of the ability to give a secondary response in vitro. The difference between these and the present results may be explained on the basis

of a more effective removal of adherent cells by the passage of spleen cells through cotton wool. This explanation seems likely because depletion of a primary response by passage through cotton was found to be more readily accomplished than depletion of the secondary response, which required two passages and therefore depended on a more rigorous cell fractionation.

The results of depletion treatments on primed rabbit spleen cell suspensions poses considerations as to the interaction between memory cells and other lymphoid cells in the secondary response in vitro. The responses to both SE and BA by primed rabbit spleen cells were approximately equal in sensitivity to depletion of adherent cells. It does not seem likely that removal of some of the primed (memory) cells was responsible for the depletion effect on the immune response, since many depleted spleen suspensions, unresponsive in dishes, performed in tube cultures as though an enrichment of memory cells had been obtained. It is less clear that the ultimate depletion of responsiveness in tube cultures was independent of a loss of memory cells, although other results (15) indicate that adherent cells isolated from the spleens, as well as thymus cells, may reconstitute the ability of depleted primed spleen cell suspensions. It should be recalled, however, that Plotz and Talal (16) demonstrated a loss of antibody-forming cells on columns, and attributed this depletion to a stickiness of blast cells. Such an adherence of blast cells might also cause a removal of a portion of the memory cells, particularly in spleen cell suspensions from recently primed animals.

The present results suggest that demonstration of the deleterious effect of removal of adherent cells on the secondary response of primed spleen cell suspensions depends on the surface area of the culture in which the immune response is induced. It seems likely that all cell-to-cell interactions needed for these in vitro immune responses can be interfered with more readily in cultures in which the cells are spread over a wider area. Similar observations were reported by others (17, 18), indicating that in cultures of peripheral blood leukocytes, DNA synthesis of cells, stimulated specifically by antigen or nonspecifically by phytoghemagglutinin (PHA), was inversely related to the surface area on which cells were maintained. The importance of intimate cell contact in the in vitro immune response has been further emphasized by the report that anti-SE PFC in mouse spleen cell cultures arise mainly in clusters (10).

Although the results of the present study appear to suggest that the proliferative response of primed spleen cells is not dependent on the presence of adherent cells, it is more likely that a quantitative difference in susceptibility to adherent cell removal may be involved. In fact, such an interpretation is supported by results of others who showed that blastogenic changes in sensitized lymphocytes of human peripheral blood were dependent on the presence of monocytes or macrophages obtained from peripheral blood (18, 20–22). In

studies with human peripheral blood cells, Valentine (18) has also shown that more purified cell suspensions were more dependent on intimate cell-to-cell interaction for proliferation in response to antigen.

Most authors agree that the proliferative response appears to represent an in vitro expression of delayed type sensitivity (23, 24). However, cellular proliferation is also an integral part of the secondary antibody response in vitro and is certainly needed in order to obtain the increased numbers in PFC observed in the culture after challenge with SE. It is unclear in the present studies to what extent the proliferative response of the spleen cells represents one or the other type of sensitization in the rabbit. It should be noted that the degree of stimulation obtained with spleen cells from rabbits primed 20 days earlier is slightly higher than was seen in previous experiments in this laboratory (25) and more similar to those obtained by Gery et al. (26).

Interpretation of the actual role of the monocyte in the antigen-specific proliferative response is not clarified, since Oppenheim et al. (22) reported that passage of human peripheral blood leukocytes on glass bead columns diminished the proliferative response of effluent lymphocytes to such nonspecific stimuli as staphylococcus filtrate and to suboptimal doses of PHA, and restoration of responsiveness was observed by addition of column-eluted monocytes. Simply increasing cell density in column-purified lymphocyte cultures also seemed to function in a restorative direction with respect to the proliferative response to both specific antigen and to PHA (22). These data again emphasize the problem inherent in all tissue culture experiments that a nonspecific trephocytic or "feeder layer" effect (27) may be as relevant in the in vitro immune response as the so-called specific "antigen processing" role assigned to cells of the monocytic series. However, the present data suggest that there is no detectable qualitative difference between the precursors of antibody-forming cells in the in vitro primary and secondary responses to particulate antigens. Precursors in both responses appear to depend on interaction with an adherent cell population for the expression of their ability to produce antibody.

SUMMARY

Both primary and secondary responses to sheep erythrocytes and to *Brucella abortus* antigen have been obtained in cultures of dispersed rabbit spleen cells. Removal of adherent cells by repeated incubation of spleen cells on absorbent cotton diminished the ability of the spleen cell suspensions to give secondary as well as primary responses in vitro.

When comparing cultures made in dishes and in tubes, the loss of responsiveness after incubation on cotton was much more evident in the dish cultures. It was concluded that the cell-to-cell interaction needed for immune responses to particulate antigens in vitro was more readily interfered with when the cells were spread over a larger surface area.

The proliferative response to antigen, as measured by uptake of ^{3}H-thymidine in tube cultures of the sensitive spleen cells, appeared particularly resistant to the depletion effect of adherent cell removal.

Dispersed spleen cells from sensitized mice gave a secondary response to sheep erythrocytes. This response was readily abolished by one incubation on absorbent cotton when the cells were cultured in dishes.

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