CELL INTERACTIONS IN THE PRIMARY IMMUNE RESPONSE IN VITRO: A REQUIREMENT FOR SPECIFIC CELL CLUSTERS*

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The interaction of two or more cell types appears to be required for the induction of the primary immune response (1–10). Clusters of lymphoid cells are apparent during the immune response in vivo and in vitro, but have not been directly related to antibody formation. Sharp and Burwell (11), and Schoenberg et al. (12) have observed cytoplasmic connections between macrophages and lymphocytic cells in vivo. The frequency of such interactions appeared to increase after antigenic stimulation. McFarland, Heilman, and Moorhead (13) have described lymphocytes interacting with macrophages by means of a "uropod" in mixed leucocyte cultures. If macrophages "process" antigen as suggested by several reports (2, 3, 14), direct cell to cell contact may be required to provide antigenic stimulation for a second cell type. The experiments reported here present evidence that most, if not all, antibody-forming cells in vitro arise in cell clusters. The interaction between cells in a cluster is specific and appears to be mediated by antigen and/or antibody on the surface of the interacting cells. Clusters are required for continued division of antibody-forming cells during the early primary response. The specific interaction of cells in clusters thus appears to be required for antibody formation in vitro.

Materials and Methods

Mice.—8–12 wk-old DBA/2 female mice obtained from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments.

Culture Reagents.—Hanks' balanced salt solution (HBSS) without bicarbonate was obtained as a 10 × concentrated solution from Grand Island Biological Co. (GIBCO), Grand Island, N. Y. Eagle's minimum essential medium (MEM) was freshly prepared for each experiment by adding to sterile glass-distilled, double-deionized water concentrated HBSS (GIBCO, No. 406), MEM amino acids (GIBCO, No. 113), MEM nonessential amino acids (GIBCO, No. 114), MEM vitamin solution (GIBCO, No. 112), sodium pyruvate (GIBCO, No. 136), L-glutamine (GIBCO, No. 503), and sodium bicarbonate to neutral pH. The medium was supplemented with 10% fetal bovine serum (Colorado Serum Co., Denver, Colo., No. 1190, Lot No. 242). A nutritional mixture for daily addition to the cultures contained twice

the normal concentration of MEM amino acids, MEM nonessential amino acids, and L-glutamine, but did not contain vitamins or pyruvate. The nutritional mixture was also supplemented with 0.01 gm per ml dextrose and adjusted to neutrality with sodium bicarbonate.

Cell Culture.—All techniques closely followed those described originally by Mishell and Dutton (15). Spleens were obtained aseptically from mice killed by cervical dislocation. From one to four spleens were placed in a 60 mm sterile disposable Petri dish (Falcon Plastics, Los Angeles, Calif., No. 1007) containing 10 ml cold HBSS without bicarbonate. The spleens were teased apart with small forceps, and the cells in suspension aspirated and transferred to a chilled 15 ml sterile disposable tube (Falcon No. 2001). The tube was placed in an ice bath and the cell aggregates allowed to sediment by gravity for 3 min. The resulting single cell suspension was transferred to a second chilled 15 ml tube and centrifuged 10 min at 600 $\times$ g at 4°C. The supernatant was discarded and the sedimented cells resuspended in medium to a concentration of 1 $\times$ 10^7 cells per ml. Each culture consisted of 1 ml of cell suspension in a 35 mm sterile disposable Petri dish (Falcon No. 1008). The Petri dishes were incubated in Plexiglas boxes in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2 (Matheson Co., Inc., Joliet, Ill.). The boxes were placed on a slowly rocking platform (Bellco, Vineland, N. J.), except where otherwise indicated. Each dish received daily 50 μl nutritional mixture and 50 μl fetal bovine serum.

Antigen.—Sheep or burro ertyhrocytes were washed three times in large volumes of HBSS and once in MEM before use as antigen. All sheep erythrocytes used were obtained from a single sheep. The burro erythrocytes were obtained (courtesy of Dr. W. M. Williamson, Chicago Zoological Park, Brookfield, Ill.) from a single burro. The antigen dose was 1 $\times$ 10^7 sheep or burro erythrocytes per culture dish. If both antigens were added to one dish, 1 $\times$ 10^7 sheep and 1 $\times$ 10^7 burro erythrocytes were used.

Antibody-Forming Cells.—Cells or cell clusters releasing "19S" hemolysins for sheep or burro erythrocytes were enumerated on the 4th day of a culture by a modification of the plaque-forming cell technique of Jerne and Nordin (16). Cell suspensions were prepared for assay by two methods. Plaque formation by cell clusters was tested using a suspension of cells aspirated from the culture dish with a large bore pipette. This sample was divided into two aliquots. The first was handled as gently as possible to prevent dispersion of cell clusters. The second aliquot was dispersed by 30 sec agitation on a vortex mixer (Super-Mixer, Lab-Line Instruments, Inc., Melrose Park, Ill.) to achieve a single cell suspension. The cell suspension to be assayed was added to a mixture of 0.5 ml 0.5% agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) and 0.05 ml 5% sheep erythrocytes at 43°C. The resulting mixture was then gently poured onto a microscope slide previously coated with 0.1% agarose. Hemolytic plaques developed during 2 hr incubation at 37°C. with 10% guinea pig serum (Markham Laboratories, Chicago, Ill.) as a source of complement. The numbers of plaques (between 20 and 200 per slide) were counted after an additional 1 hr incubation at 4°C using 10 magnification and an electronic scoring device. Two slides were prepared from each culture: one from nondispersed cells for the detection of hemolytic plaque-forming clusters and one from dispersed cells for the detection of individual plaque-forming cells. Each number in each table is the mean of plaque counts on at least four slides expressed as plaques or plaque-forming cells per 1 $\times$ 10^6 cells at the beginning of culture.

In some experiments, both sheep and burro erythrocytes were added to the same culture. Cells from such cultures were assayed for plaque-forming ability against sheep erythrocytes, burro erythrocytes, and a mixture of both. The assay procedure was the same as previously described. In the case of burro erythrocytes, 0.05 ml of a 5% suspension was incorporated into the agarose layer. With the mixture of antigens, 0.05 ml of 2.5% sheep and 2.5% burro erythrocytes was added to the agarose-cell suspension. Plaques were developed with guinea pig complement as before. When cells or cell clusters were assayed against the erythrocyte mix-

ture, plaques were scored as "clear" (very few erythrocytes remaining within the area of the plaque), or "cloudy" (many erythrocytes remaining within the confines of the plaque). Cloudy plaques could be converted to clear plaques by lysing one type of erythrocyte with specific antiserum. Cloudy plaques contained many more residual erythrocytes than any plaques found when cells were assayed against a single antigen.

Antiserum.—Antibody to sheep or burro erythrocytes was prepared by injecting 2×10^8 erythrocytes intraperitoneally into DBA/2 mice. 1 wk later, a similar injection was given. After 1 more wk, the mice were bled by cardiac puncture and the serum obtained. Normal mouse serum was collected from unimmunized animals. Sera were pooled, sterilized by passage through a prewashed Millipore filter (0.42 μ, Millipore Filter Corp., Bedford, Mass.), and stored frozen until use. High concentrations of normal mouse serum may prevent the in vitro immune response, but dilutions of 1:100 as used in these experiments had no effect on the response.

Autoradiography.—Cells in culture were labeled by exposure to 0.5 μC ^{14}C-thymidine (Schwarz BioResearch, Orangeburg, N. Y.) for 1 hr. Slides for the detection of plaque-forming cells and clusters were prepared and plaques developed as previously described. The slides were fixed in 10% buffered formalin in HBSS and the salts leached out of the agarose by several changes of distilled water. The slides were dried at room temperature and coated with Kodak NTB-2 emulsion. The radioautographs were developed after 2 wk exposure and the cells stained with hematoxylin and eosin. The number of silver grains was counted under magnification of 950.

Several control procedures were done. Slides were treated with 250 μg per ml DNase (Worthington Biochemicals, St. Louis, Mo.) at 37°C. for 1 hr prior to fixation. Some slides were exposed to 10% trichloroacetic acid (TCA) at 4°C. for 1 hr after drying. The number of silver grains was not changed by cold TCA treatment, but was markedly reduced by DNase treatment indicating that the labeled thymidine had been incorporated into DNA.

PRELIMINARY OBSERVATIONS

Spleen cell cultures examined 4 days after in vitro stimulation with sheep erythrocytes contained many clusters of cells. Cell clusters contained up to 100 cells, but most clusters contained fewer than 20 cells. Clusters included large monocytic cells and many lymphocytes. That most plaque-forming cells arise in cell clusters was shown in the following experiments.

Antibody production by nondispersed and dispersed cells was assayed on the 4th day of culture. The cell suspension to be assayed was taken directly from a culture dish without scraping free those cells adhering to the bottom of the dish. This modification of the usual assay technique was necessary because scraping the culture dish resulted in the dispersion of many cell clusters. The number of plaque-forming cells adherent to a culture dish was small compared to the total number of plaque-forming cells in a culture; for example, dispersed cells from cultures not scraped produced an average of 120 plaques per 10^6 cells initially cultured, while dispersed cells from cultures scraped to recover all cells produced an average of 132 plaques.

In 12 replicate experiments (Table I), nondispersed cells from the culture supernatant produced an average of 30 plaques per 10^6 cells initially cultured, and dispersed cells produced an average of 173 plaques per 10^6 cells. Most

plaques produced by nondispersed cells contained a central cluster of cells and nearly every plaque produced by dispersed cells contained a single cell in the center. These results indicate that each cluster that formed a plaque contained an average of about six individual plaque-forming cells. Only about 10% of all clusters, however, formed plaques against sheep erythrocytes. Since it is known (17–19) that most antibody-forming cells arise by division, it was presumed that cells in some of the clusters should be undergoing rapid division. This possibility was confirmed in the following two experiments.

TABLE I

Plaque Formation by Nondispersed and Dispersed Spleen Cell Suspensions on the 4th Day of the Primary In Vitro Response to Sheep Erythrocytes

Experiment	Before dispersion*	After dispersion*
1	14‡	75§
2	22	193
3	21	146
4	5	180
5	16	221
6	8	120
7	110	346
8	34	279
9	28	97
10	77	205
11	14	98
12	8	112
Mean ± S.E. of the mean	29.75 ± 2.72	172.7 ± 6.60‖

* Each number is the mean of four plaque counts on cells from four replicate cultures.
‡ Plaques per 10^6 cells at the beginning of culture.
§ Plaque-forming cells per 10^6 cells at the beginning of culture.
‖ The means of 29.75 and 172.7 are significantly different from each other (P < 0.05).

Velban, a mitotic inhibitor, was added to cells on the 4th day of culture. 6 hr later, cultures were harvested and the number of cells in metaphase determined. About 10% of the clusters contained nearly all the cells in mitosis, and some of these clusters contained as many as 10 metaphase figures. In cultures not stimulated with antigen, many fewer cells were in metaphase.

A second experiment was carried out to establish that the rapidly dividing cells were also the antibody-forming cells. On the 4th day of culture, spleen cells immunized in vitro were labeled for 1 hr with thymidine-^{14}C. Cell clusters were assayed for plaque formation as usual, and the slides prepared for autoradiography after plaques had appeared. When the slides were developed, all clusters which had formed plaques contained several cells with a heavily-labeled

nucleus. The same clusters contain both rapidly dividing cells and antibody-forming cells, which, from other evidence (20), are presumed to be the same cell. The special microenvironment of a cell cluster seems to be required for the primary immune response in vitro. The requirement for cell clusters and the mechanism of specific cell aggregation to form clusters were examined in the main set of experiments.

EXPERIMENTAL OBSERVATIONS

The Effect of Preventing Cell Clusters.—It was possible to examine the immune response in vitro with and without cell clusters by taking advantage of the observation that when cell clusters are dispersed, reaggregation occurs only if

TABLE II

Plaque-Forming Cell Response of Spleen Cultures with and without Cell Clusters

Day 2 treatment*	Day 4 response			
	Cell clusters	Plaque-forming cells‡		
		Exp. 1	Exp. 2	Exp. 3
Cells dispersed, culture stationary	Absent	8	2	8
Cells dispersed, culture rocking	Present	207	180	192
No dispersion, culture stationary	Present	201	166	198
No dispersion, culture rocking	Present	277	192	188
Background, no antigen	Present	2	2	4

* No rise in numbers of plaque-forming cells had yet occurred on day 2.

‡ Plaque-forming cells per 10^6 cells initially cultured.

the single cell suspension is cultured on a rocking platform. Gentle rocking presumably promotes interaction of cells. Cells in clusters fail to reaggregate if a dispersed cell suspension is left stationary. The following experiment demonstrated that the integrity of cell clusters is required for the in vitro response to sheep erythrocytes.

Cell cultures were stimulated with antigen and cultured as usual on a rocking platform for 2 days. Several cultures were then assayed for plaque-forming cells and found to contain only the background number, that is, at 2 days no significant increase in plaque-forming cells had occurred. The remaining cultures were separated into two groups and treated as follows: the first group of cultures was dispersed by mechanical agitation so that each culture contained a suspension of single cells; half of this group was cultured from day 2 to day 4 on a stationary shelf, and the other half of the group was returned to the rocking platform. The second group of cultures was not dispersed, but was either cultured in a stationary position for 2 more days, or was rocked in the usual

fashion. On day 4, the plaque-forming cell response of all cultures was determined, and a number of cultures were examined by phase microscopy to estimate the extent of cell clustering. The results of three such experiments are presented in Table II. If cultures were dispersed and kept stationary, most clusters did not reform and the plaque-forming cell response was abolished. When the dispersed cells were allowed to reaggregate by rocking the cultures, clusters appeared in the usual size and numbers, and the plaque-forming cell response was normal in magnitude. Leaving nondispersed cells stationary did not reduce the magnitude of the response, nor was clustering changed. The clusters that reformed after dispersion of cells on day 2 contained as many plaque-forming cells as those clusters which had formed during the first 2 days of culture and had not been dispersed. It was only in the experimental situation that prevented cell clustering that the proliferation of antibody-forming cells failed to occur. Cell clusters, therefore, appear to be required for the immune response in vitro.

Specificity of Cell Clusters.—If cell clusters have a specific immunological function, then each cell cluster should contain only one type of antibody-forming cell, regardless of the number of antigens present. If, on the other hand, some cells in clusters have a general nonspecific function (e.g., a "nurse cell"), or antibody-forming cells in general have some property which causes them to cluster, then several types of antibody-forming cells might appear in one cluster when cultures were stimulated with several antigens. The following experiments demonstrated that one cell cluster contains one type of antibody-forming cell.

Sheep and burro erythrocytes were added to the same culture. It was known from preliminary experiments that the in vitro response to sheep and burro erythrocytes was of the same order of magnitude, and that simultaneous addition of both erythrocytes to one culture did not diminish the response to either antigen. 4 days later, the plaque-forming cell response of cell clusters was measured against a mixture of antigens, as well as against each erythrocyte alone. When cell clusters were tested against the erythrocyte mixture, any cluster containing both types of antibody-forming cells should lyse both erythrocytes to produce a "clear" plaque. No such clear plaques were found; that is, all plaques formed by clusters assayed against both antigens contained many residual erythrocytes (see Table III, part a). If all the sheep erythrocytes on a slide were lysed with anti-sheep erythrocyte antibody, a smaller number of clear plaques were left, which number was approximately equal to the number of plaques formed by clusters tested against burro erythrocytes alone. These results support the conclusion that each cluster contained cells forming anti-sheep or anti-burro erythrocyte antibody, but not a mixture of cells forming both types of antibody. Unused portions of the cultures from this experiment were used for the following experiment.

Specific Reaggregation of Cell Clusters.—A suspension of plaque-forming cells will reaggregate to form cell clusters. Reaggregation might be "specific"; that is, only cells forming antibody of the same specificity could reaggregate, or "nonspecific," with all antibody-forming cells sharing some general property that causes them to reaggregate.

After sampling cultures for the previous experiment, the remaining cells were dispersed and the cultures returned to the rocking platform for 1 hr, at the end of which time reaggregated clusters were microscopically evident. These reaggregated cells were tested for plaque formation against a mixture of sheep and burro erythrocytes, and against each antigen separately. The results

TABLE III

Antigen-Specific Reaggregation of Dispersed Spleen Cells In Vitro

Plaques*	Exp. 1	Exp. 2	Exp. 3
Before dispersion of cells in culture			
Nondispersed cell aliquot	0	0	0 clear
	13	28	38 cloudy
Dispersed cell aliquot	0	0	1 clear
	99	91	193 cloudy
After reaggregation of cells in culture			
Nondispersed cell aliquot	0	0	0 clear
	19	24	49 cloudy
Dispersed cell aliquot	3	0	0 clear
	123	104	136 cloudy

* Plaques per 10^6 cells initially cultured.

are shown in Table III, part b. As before, clusters produced only "cloudy" plaques, and thus appeared to contain only cells forming antibody to one erythrocyte antigen. The number of plaque-forming cells per cluster was similar to the number before dispersion and reaggregation. These results rule out nonspecific reaggregation, since the probability of a cluster containing several cells forming antibody of one specificity after random reaggregation is very small. Randomly reaggregated cells should have contained cells forming antibody to sheep erythrocytes and cells forming antibody to burro erythrocytes. Cells in clusters appear, then, to have a specific means of reaggregation. The relationship of antibody and clustering was examined in the next set of experiments.

Effect of Antibody on Cluster Reaggregation.—If antibody produced by cells in clusters provides the means for clustering, then dispersing the clusters and

adding an excess of exogenous antibody might prevent reaggregation of clusters either by (a) blocking antigen or antigenic fragments involved in clustering, (b) passively adsorbing to neutral cells thereby causing the formation of immunologically "sterile" clusters, or (c) saturating the "binding sites" available on the surface of the interacting cells. It was not possible to directly assess the action of antibody on cell clusters, since the 10 % of all clusters involved in producing antibody to sheep erythrocytes provided too small a sample to accurately determine reduction of numbers of clusters. It was possible, however, to demonstrate indirectly that antibody prevented cluster reaggregation.

Cell cultures were stimulated with sheep erythrocytes and cultured for 2 days as usual. At this time, few, if any, plaque-forming cells had developed.

TABLE IV

Inhibition of the Plaque-Forming Cell Response and Cluster Reaggregation by Specific Antibody

Day 2 treatment	Day 4 response		
	Plaque-forming cells*		
	Exp. 1	Exp. 2	Exp. 3
Cells dispersed, culture stationary	31	8	16
Cells dispersed, culture rocking	124	202	108
Cells dispersed, antibody‡, culture rocking	28	10	12
No dispersion, culture stationary	108	166	143
No dispersion, culture rocking	123	220	187
No dispersion, antibody‡, culture rocking	112	175	166

* Plaque-forming cells per 10^6 cells initially cultured.

‡ 1/100 diluted mouse anti-sheep erythrocyte serum was added to cultures for either 1 hr or 2 days with the same results (see text).

The cultures were divided into two groups; the first group was dispersed, and the second group was not dispersed. Cultures in the first group were either left stationary from day 2 to day 4, returned to the rocking platform, or treated with a 1 to 100 dilution of isologous anti-sheep erythrocyte serum, anti-burro erythrocyte serum, or normal serum. Some of the cells were incubated with the sera for 1 hr and then washed three times with medium before being returned to the rocking platform. In other cultures the sera remained during the last 2 days of culture. The cultures in the nondispersed group were either left stationary, rocked as usual, or exposed to the above sera for 2 days. The plaque-forming cell response of clusters and dispersed cells was measured on day 4 of culture. Those cells still in the presence of anti-serum were washed three times after harvesting to remove excess antibody. In several separate slides, plaques were eliminated by treating cells with a metabolic inhibitor, rotenone, indicating

that plaque formation was not due to passive adsorption of antibody to cells (21). The results of this experiment are given in Table IV. As before, the plaque-forming cell response was abolished by dispersing cell clusters and preventing them from reforming by keeping the cultures stationary. Adding anti-sheep erythrocyte antibody to dispersed cells (either for 1 hr or for 2 days) had precisely the same effect, although the cultures had been maintained on a rocking platform. Antibody added to dispersed cells on day 2 blocked the response as measured 2 days later. On the other hand, adding antibody to intact cell clusters did not diminish the plaque-forming cell response. Addition of anti-burro erythrocyte serum or normal mouse serum to dispersed cells or clusters had no effect on the final number of plaque-forming cells. The effect of adding antibody to the antigen was exactly the same as mechanically preventing cluster reaggregation; therefore, it seems probable that the effect of antibody was to prevent the reaggregation of those clusters containing antibody-forming cells.

DISCUSSION

We have recently presented evidence that at least two and possibly three different cell types are required to interact during the in vitro response of mouse spleen cells to sheep erythrocytes (1, 22). Spleen cells which adhere to plastic and spleen cells which do not adhere are both required for the in vitro response. The adherent cell population contains the cells which first interact with antigen. The nonadherent cell population contains one and probably two populations of cells which must interact with the adherent cells before the proliferation of antibody-forming cells occurs. Only small subpopulations of adherent and nonadherent cells appear to interact during the response to one antigen. The immune response in vitro is thus more complex than has been generally thought, and the interpretation of the present work must reflect this complexity.

Cells in clusters must interact one with another for the continued proliferation of antibody-forming cells. If clusters of cells were dispersed and prevented from reaggregating, either by mechanical means or by excess antibody, the plaque-forming cell response was blocked.

Antibody-forming cells are in clusters. It is not known whether plaque-forming cells cluster only with themselves or whether several cell types interact in clusters to stimulate and regulate the proliferation of antibody-forming cells. It is possible that some cells in clusters have some general function unrelated to either antigen uptake or antibody synthesis, but essential for rapid division of antibody-forming cells. Morphologically, clusters contained several cell types. All clusters contained small lymphocytic cells and many, but not all, contained one or more larger monocytic cells. The interpretation of morphology was hampered because an unknown number of cells may have been

dispersed during the preparation of stained smears, and the cells remaining in clusters were often superimposed one on another. Ideally, only the clusters containing plaque-forming cells should be evaluated and, therefore, we have examined clusters after assay by the Jerne technique; however, cellular detail of cells fixed, dried, and stained in agarose was poorly preserved. Thin sections of clusters for light and electron microscopy may provide better understanding of the relationship of cells in clusters and are being prepared.

Antibody-forming cells reaggregate specifically in clusters. Antibody prevents the proliferation of antibody-forming cells, apparently by preventing reaggregation of cells in clusters. Specific interaction of cells may be mediated by antibody and antigenic determinants on cell surfaces. The presence of immunoglobulins on the surface of cells has been reported (23-25). Antigen has recently been demonstrated in association with the surface of cells (26). Mitchison, working with hapten inhibition of the response to hapten-protein conjugates, has postulated that immunocompetent cells bear antibody on their surfaces and that combination of antigen with the antibody sets off the events leading to production of the same antibody by many cells (27).

Cells could interact via antibody and antigenic determinants in several ways. One cell with an antigenic determinant on its surface might interact with antibody bound to the surface of a second cell. Or two cells with antigenic determinants on their surfaces might interact with antibody serving as a ligand. The opposite situation is also possible; that is, two cells with antibody fixed to their surfaces might interact via antigen. All of these means of interaction would be blocked by excess extracellular antibody.

The specific interaction of cells is required for differentiation in several biological systems (28, 29). The similarity between such interactions and clustering during the immune response is striking.

SUMMARY

Mouse spleen cells were found to associate in cell clusters during the primary immune response to sheep erythrocytes in vitro. About 10% of the cell clusters had the following unique properties; (a) they contained most, if not all, antibody-forming cells, (b) they contained only cells forming antibody to one antigen when cell cultures were immunized with two antigens, (c) the cells in clusters reaggregated specifically after dispersion, and (d) the specific reaggregation of clusters appeared to be blocked by antibody to the antigen. The integrity of cell clusters was required for the proliferation of antibody-forming cells, and prevention of clustering by mechanical means or by excess antibody blocked the immune response. Antibody and antigenic determinants on the surfaces of cells probably provide the basis for interaction. The unique microenvironment of cell clusters was essential for the primary immune response in vitro.

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