



## **Immune Response Restoration with Macrophage Culture Supernatants**

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## Immune Response Restoration with Macrophage Culture Supernatants

**Abstract.** *Depression of the in vitro immune response of mouse spleen cell suspensions to sheep erythrocytes by removal of macrophages can be reversed by the addition of supernatants from peritoneal macrophage cultures. Supernatant activity can be absorbed by the red cell antigen, and supernatant-treated red cells are stimulatory in the absence of macrophages or supernatant.*

Cell suspensions from the spleens of normal mice are no longer able to develop an immune response to foreign erythrocytes if a subpopulation of cells which attach to glass or plastic surfaces is first removed (1, 2). Similar effects have been shown with spleen cells from rabbits (3). The ability to respond is restored when attached and nonattached cell populations are recombined. Since the precursors from which the antibody-forming cells are derived are present in the nonattached cell population (2, 4), the attached cell population must provide a "helper" effect, which is necessary for some step or steps in the series of events that lead to the development of a population of antibody-forming cells. The attached cells from the spleen are phagocytic, and their function can be replaced by peritoneal macrophages. A number of roles has been proposed for macrophages in the immune response (including antigen concentration, antigen processing, or the provision of informational RNA for use by lymphocytes). Although there is evidence in support of each of these hypotheses there is still some question whether the problem has been fully resolved. We now describe experiments on the role of the attached cell population with the use of macrophages from the peritoneal cavity (5), which show that the macrophage function can be replaced by a soluble factor obtained from macrophage culture supernatants (6).

Mice 2 to 3 months old (C57BL/6 × DBA/2 F<sub>1</sub> hybrids) were used in all experiments. Sheep red blood cells (SRBC) and burro red blood cells (BRBC), which show less than 1 percent cross-reactivity, were used as antigens.

The various cell suspensions were prepared from normal mice and cultured with antigen in a modified Eagle's medium containing 5 percent fetal bovine serum (FBS) (7). Peritoneal cells were harvested from the peritoneal cavities of normal mice by injection and withdrawal of medium without serum (5). Attached cells were prepared by incubating peritoneal cells in culture dishes and removing the nonattached

cells after 2 hours (5). The culture dishes with the attached cells were then used for the culture of the nonattached cells or for the preparation of supernatant. Nonattached spleen cells were prepared by allowing the attached cells to adhere to glass beads (2). The nonattached cell suspension was removed; the cells were recovered by centrifugation, washed, and finally resuspended in complete medium. The additional washing step resulted in more effective depletion of the response. The standard antigen dose of 30 μl of a 1 percent (by volume) suspension of erythrocytes was used in all cases and was added at the start of incubation. (This dose is slightly in excess of the smallest dose required to give a maximum response.) The number of antibody-forming cells was assayed on days 4, 5, and 6 by the Jerne direct hemolytic plaque assay; the results are expressed as the number of plaque-forming cells (PFC) per 10<sup>6</sup> recovered cells.

Removal of the attached cell population from the whole spleen cell suspension results in a marked reduction in the number of antibody-forming cells recovered at day 5 (Table 1) (1). The response of the nonattached cell population is restored by the addition of peritoneal cells. The peritoneal cells can be separated into an attached and nonattached cell population, and it is the attached cells that have restorative activity. Restoration of the response was also seen if 24-hour culture supernatants from the incubation of attached

cells were added to cultures of nonattached cells. In these experiments approximately 1 × 10<sup>6</sup> peritoneal attached cells or, more usually, unfractionated peritoneal cells from normal mice were incubated in 4 ml of complete medium without FBS at 37°C for 24 hours. No antigen was added. At the end of the incubation period, the cultures were harvested and the cells were removed by 20 minutes centrifugation at 1000g at 4°C for 20 minutes. Varying amounts of supernatant were added to nonattached spleen cells suspended in complete medium, and antigen was then added. In every case, the presence of supernatant enhanced the response of nonattached cells (Table 1). In some cases (not shown) high concentrations of supernatant were less stimulatory than lower concentrations. This was not realized in the initial experiments (6) and may account for the fact that some supernatant preparations at that time appeared to have little activity.

The activity of the supernatants was not removed by mild heat (57°C for 30 minutes) or by passage through a 22-μm pore filter. The activity was not stable to freezing and thawing unless serum was present in the medium.

The restoration activity could be partially removed from the supernatant by absorption with sheep erythrocytes. In these experiments, 2 ml of supernatant were incubated with 0.5 ml of packed erythrocytes for 2 hours at 4°C (Table 2). It seemed that there was no antigen specificity in the absorption of supernatant, but the results were not conclusive on this point. Thus SRBC usually removed the supernatant activity for BRBC and vice versa (data not shown), but this was not always true, for example, experiment 1, Table 2. It seems likely that erythrocyte antigen is solubilized during the absorption treatment, and this produces a stimulatory

Table 1. The restoration of the responsiveness of nonattached spleen cell suspensions by the addition of peritoneal cells or peritoneal cell culture supernatants. Whole spleen cell suspensions were cultured at 1 × 10<sup>7</sup> cell/ml; nonattached cells were cultured at 2 to 3 × 10<sup>6</sup> cell/ml, and peritoneal cells at 1 × 10<sup>5</sup> cell/ml. Supernatants (SN) were prepared as described in the text and diluted to the final concentrations in the cultures as indicated. All cultures were stimulated with and assayed with SRBC.

Cell cultures		PFC/10 <sup>6</sup> recovered cells assayed on day 5				
Cell suspensions	Additions	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Whole spleen		980	780	590	681	
Nonattached		12	82	8	55	58
Nonattached	SN/3	1388	890			
Nonattached	SN/10	2500	995	879	2531	889
Nonattached	SN/30	1142	1180	619	1280	295
Nonattached	SN/90	477	1215		618	36
Nonattached	SN/270		910			
Nonattached	Peritoneal cells		2730	3195		

Table 2. The absorption of supernatant activity with SRBC. Supernatant were prepared and absorbed (abs) with SRBC as described in the text. Cultures (nonattached spleen cell suspension) challenged with SRBC were assayed against SRBC, cultures challenged with BRBC were assayed against BRBC (10).

Additions to cultures	PFC/10 <sup>6</sup> recovered cells assayed on day 5		
	Exp. 1	Exp. 2	Exp. 3
SRBC	65	65	107
SN/10 plus SRBC	1206	1275	1150
SN/10 abs plus SRBC	640	805	666
SN/10 abs	375	708	787
BRBC	0	0	0
SN/10 plus BRBC	152	85	182
SN/10 abs plus BRBC	127	0	8

effect for that particular antigen which tends to obscure the reduction due to removal of the supernatant activity.

Erythrocytes that have been incubated in attached cell supernatant have, in some way, acquired the ability to stimulate a response in nonattached spleen cell populations in the absence of either attached cells or supernatant (Table 3). In these experiments, 0.2 ml of 10 percent erythrocytes was incubated in 2 ml of various dilutions of supernatant for 60 minutes at 37°C. The erythrocytes are washed two times in 12 ml of balanced salt solution before final resuspension in medium. Thirty microliters of a 1 percent (by volume) suspension of erythrocytes treated in this way were added to the cultures of nonattached cells.

Erythrocytes treated in high concentrations of supernatant were less stimulatory than those treated in diluted supernatant. It was consistently observed that much lower concentrations of supernatant were effective for the treatment of erythrocytes than was the case when supernatant was added directly to the cultures. It is possible that the absorption or interaction of the supernatant with the erythrocytes occurs

more efficiently in the absence of serum and other cells that are present when supernatant is added to the culture.

The experiments presented in Table 1 show that supernatants obtained from the incubation of peritoneal cells will enhance the response of nonattached cells to erythrocyte antigens, and active supernatant can be generated in the complete absence of antigen.

Attached cells are generally more active than supernatant in restoring the response. This may be because they produce supernatant factor continuously in optimum amounts or it may be due to some other reason. Whether supernatant will completely substitute for the cells or whether other cell functions are involved is not clear.

The following possibilities for the nature of supernatant activity can be considered: (i) That it is antibody with specificity for determinants of the erythrocyte antigens, (ii) that it is an enzyme whose interaction with the erythrocyte membrane is necessary for immunogenicity, and (iii) that it represents some nutritional or hormonal factor necessary for cells of the nonattached cell population to function in the immune response.

Table 3. Stimulation of nonattached cells with supernatant-treated erythrocytes ( $3 \times 10^6$  cells per culture). Nonattached cells ( $2$  to  $3 \times 10^6$ ) were incubated with untreated SRBC, untreated SRBC plus dilutions of supernatant, or with SRBC treated with various dilutions of supernatant. Supernatants were prepared as indicated in the text. N.T., no treatment.

Dilution of supernatant		PFC/10 <sup>6</sup> recovered cells assayed on day 5				
Added to cultures	For treatment of SRBC	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
	N.T.	160	160	21	190	130
1/10	N.T.	645	475	314	469	666
1/30	N.T.	662	527	415	1608	1550
1/100	N.T.	470	619	110		941
	1/25	835	314			
	1/100-1/125	1173	856			145
	1/200			11		714
	1/400			131	1190	878
	1/500			208		
	1/600-1/625	952	1342	47	386	
	1/800				529	785
	1/1600				210	392
	1/3200			30		330
	1/6400			18	182	87

The subsequent experiments do surprisingly little to distinguish between these possibilities. The fact that the activity can be absorbed by erythrocytes and that the supernatant-treated erythrocytes become active strongly suggests an interaction between supernatant factor and the red cell membrane, and this interaction does not appear to be antigen specific. Antibodies are not ruled out however, because they might bind to red cells through sites present on their Fc regions. Treated red cells, however, were not agglutinated by antiserum to IgG immunoglobulins or by antiserum to kappa light chains.

A curious aspect of supernatant activity is the lack of effect at high concentrations. Either a second inhibitory factor must be present or supernatant itself is inhibitory when present in excess.

Bach *et al.* (8) have reported an activity in the culture supernatants of attached cells which will restore the ability of human peripheral leukocyte populations, depleted of all macrophagic cells, to respond to antigen by increased thymidine uptake. McNeill (9) has described another factor in serum which enhances the growth of bone marrow colonies in the presence of antigen. The relation between these factors and the supernatant factor described here is unknown.

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#### References and Notes

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10. Sheep red blood cells were obtained from the Colorado Serum Co., and burro red blood cells were obtained from the Davis Laboratories.
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