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Peritoneal Macrophages in the Immune Response to SRBC *in vitro*

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Summary. The role of macrophages in the immune response against SRBC in spleen cell suspension cultures obtained from mice was studied.

1. The presence of macrophages increases the number of PFC against SRBC in spleen cell cultures.

2. The ratio peritoneal exudate cells to spleen cells is very critical. Two $\times 10^5$ peritoneal exudate cells preincubated with sheep red blood cells stimulate 1×10^7 mouse spleen cells to an optimal response. This reaction is suppressed when 2×10^6 peritoneal cells are used.

3. Spleen cell populations which had lost the capability of producing antibodies by elimination of cells adhering to glass yielded a strong immune response upon addition of 2×10^5 peritoneal exudate cells. The significance of macrophages for the induction of the immune response is discussed.

INTRODUCTION

During the primary immune response in mice the co-operation of different cell types is necessary. Cell transfer experiments in irradiated mice have shown that an immune response to sheep red blood cells (SRBC) occurs only if either thymus cells or thoracic duct cells are simultaneously transferred with bone-marrow cells (Miller and Mitchell, 1968; Mitchell and Miller, 1968). *In vitro* experiments suggest that for the primary immune response to SRBC three different cell types are required (Mosier and Coppleson, 1968). One of these cell types shows macrophage properties, i.e. phagocytosis and adherence to glass.

The function of macrophages in the primary immune response has been intensively investigated. Some results (Frei, Benacerraf and Thorbecke, 1965; Nossal, Ada and Austin, 1965; Dresser and Mitchison, 1968) suggest that antigens can induce an immune response only via macrophages, whereas direct contract between antigens and lymphocytes causes paralysis. Protein antigens are remarkably more effective when macrophage-bound than in free form (Unanue and Askonas, 1968; Mitchison, 1969). On the other hand, suppressive effects on the immune response to sheep red blood cells in mice have been ascribed to macrophages (Perkins and Makinodan, 1968).

In the present paper the role of macrophages from the peritoneal cavity in the immune response to SRBC induced in mouse spleen cell cultures is investigated.

MATERIALS AND METHODS

Animals

Female inbred NMRI mice weighing 20–25 g were obtained from the Zentralinstitut für Versuchstierzucht, Hannover.

Cell cultures

The cultivation of spleen cell suspensions was performed according to Mishell and Dutton (1967). To obtain macrophage cultures, peritoneal exudate cells (PEC) were taken from the peritoneal cavity of untreated mice and suspended in 2·5 ml of the same medium used for spleen cultures. The cell titre determined in a haemocytometer ranged from 1·8 to 3×10^6 cells/ml. PEC were seeded in Falcon Petri-dishes (diameter 35 mm) at different cell concentrations (in 1 ml) and were incubated with and without 3×10^6 sheep erythrocytes respectively for 20 hours at 37°. Before the addition of the spleen cells the peritoneal cell cultures were washed 5 times with 1 ml Balanced Salt Solution and once with 1 ml Eagle's Minimal Essential Medium to remove those cells not adhering to the dish as well as free SRBC. Usually approximately 50–60 per cent of the PEC remained on the glass as a highly pure macrophage population. To this 1 ml of spleen cell suspension was added. Since it was impossible to determine the exact concentration of the remaining macrophage fraction, all the data of PEC concentrations refer to the number of originally seeded peritoneal exudate cells.

Identification of antibody-producing cells (PFC)

PEC were removed from the dishes with a plastic scraper and washed. The number of antibody-forming cells detectable in the direct plaque test was determined according to Jerne and Nordin (1963). The plaque count was related to 10^6 harvested cells counted in the Coulter Counter (PFC/10^6 cells).

Separation of spleen cells into cells adhering to glass and cells not adhering to glass

10^7 spleen cells were incubated at 37° in Falcon dishes together with 1·6g glass beads (diameter 0·6 mm) without agitation for 30 minutes in Eagle's Minimal Essential Medium, prepared as above. Subsequently the non-adhering cells were carefully removed with a Pasteur-pipette and collected for further use. The cell titre thus obtained varied from 3·1 to $4{\cdot}4\times10^6$ cells/ml.

RESULTS

PEC-cultures were incubated at different concentrations with 3×10^6 SRBC at 37° After 20 hours the non-adhering cells as well as free antigen were carefully washed off and the remaining cells were covered with 1×10^7 spleen cells without an additional supply of antigen. 3×10^6 SRBC were added to spleen cultures cultivated for control purposes without macrophages. The results are shown in Fig. 1. On day 3 the control cultures had an average of 200 PFC, on day 4 they had 550 PFC/10^6 cells. A similar immune response was induced in spleen cells by about 5×10^5 PEC whereas 2×10^6 PEC almost completely suppressed this reaction. Optimal results (doubling of PFC) were obtained when 2×10^5 PEC, preincubated with SRBC, were combined with 10^7 spleen cells.

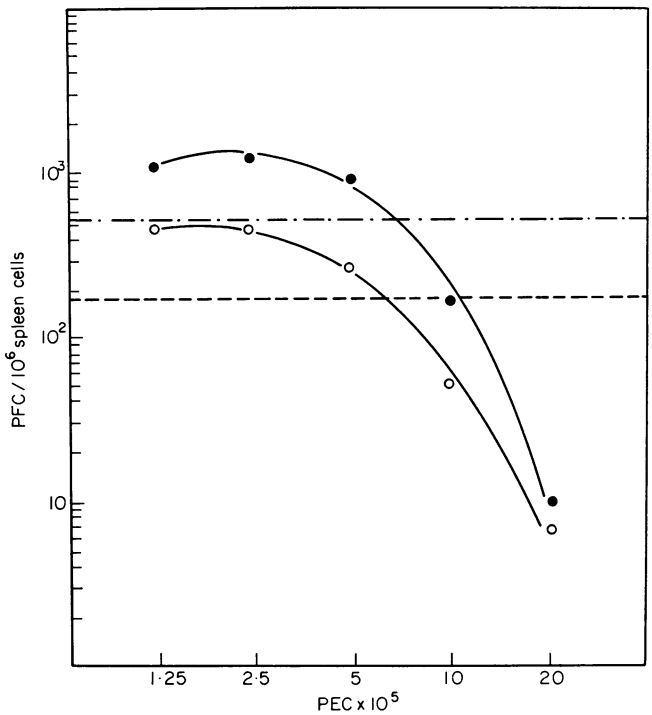

FIG. 1. Immune response *in vitro* to SRBC after combining different amounts of PEC preincubated with SRBC and 1×10^7 spleen cells. -○- day 3 in culture; -●- day 4 in culture; - - - control without PEC day 3; —·— control without PEC day 4.

The results obtained by combining different numbers of peritoneal cells with 10^7 spleen cells are shown in Table 1. PEC were incubated for 20 hours with and without antigen washed 6 times and combined with spleen cells. Spleen cells, without addition of PEC and after being cultivated with SRBC, produced an average number of 520 antibody-forming cells within 4 days. This number increased 3–4 times after combination with 2×10^5 PEC, regardless of whether the macrophages had been preincubated with antigen or not. Addition of 2×10^6 PEC almost completely suppressed the immune

TABLE 1

IMMUNE RESPONSE TO SHEEP RED BLOOD CELLS IN SPLEEN CELL SUSPENSION CULTURES AFTER ADDITION OF DIFFERENT NUMBERS OF PEC

Cell combination	PFC per 10^6 harvested cells		
	day 3	day 4	day 5
spleen cells + SRBC	152	520	215
2×10^5 PEC + spleen cells + SRBC	540	1700	1000
2×10^5 PEC + SRBC + spleen cells + SRBC	510	1900	1360
2×10^6 PEC + spleen cells + SRBC	10	15	–
2×10^6 PEC + SRBC + spleen cells	7	10	–
spleen cells	35	29	11
2×10^5 PEC + spleen cells	180	270	80
2×10^5 PEC + SRBC + spleen cells	520	1280	610
2×10^5 PEC + SRBC	0	0	0

response. It was striking that no transformation into blast cells, proplasma cells, and plasma cells took place in those cultures with a high dose of macrophages, whereas the assay referred to in lines 2, 3, 7 and 8 showed up to 50 per cent of such transformed immunocytes on day 4. In normal spleen cultures the maximum amount of transformed cells, as determined by their size, was 20 per cent.

Spleen cell cultures form antibodies against SRBC antigens even without addition of SRBC to the cultures. This fact seems to be due to antigens detectable in calf serum precipitating with SRBC-antiserum (Mishell and Dutton, 1967). The number of cells forming antibodies against these serum antigens was much lower than in cultures with addition of SRBC. This number could be increased from 29 to 270 PFC on day 4 by adding 2×10^5 PEC.

Line 8 shows an immune response of the spleen cells induced by PEC-bound antigen. Although only PEC were incubated with SRBC in these cultures, the proliferation of antibody-forming cells up to day 3 took place to almost the same degree as in combined cultures in which the spleen cells had contact with additional free antigen. From day 3 and 4 of cultivation onward the increase was less.

To test whether the described PEC response is specific to the antigens used, macrophages of one set of cultures were preincubated with 3×10^6 SRBC, those of another set with 3×10^6 burro red blood cells (burro-RBC). On combination with spleen cells one half of the cultures of each set was additionally supplied with 3×10^6 SRBC. The results of the plaque-tests of day 3 and day 4 are shown in Table 2. The immune response of spleen cells to burro-RBC alone was less strong than to SRBC. On day 4 170 PFC/10^6 cells could be detected. Antigens in calf serum stimulated cultures to form antibodies which reacted with antigens of SRBC (see Table 1), but not with antigens of burro-RBC. Preincubation of PEC with burro-RBC resulted in a clear immune response of co-cultivated spleen cells to burro-RBC, but had no influence on the antibody formation against SRBC in these suspensions. These findings suggest a co-operation of macrophages with spleen cells in the immune response.

TABLE 2

CO-OPERATION OF PEC WITH SPLEEN CELLS IN THE IMMUNE RESPONSE TO BURRO RBC OR SRBC

Cell combination	PFC per 10^6 harvested cells tested against RBC			
	day 3		day 4	
	Sheep	Burro	Sheep	Burro
2×10^5 PEC + SRBC + spleen cells + SRBC	300	0	1950	0
2×10^5 PEC + SRBC + spleen cells	275	0	970	0
2×10^5 PEC + Burro RBC + spleen cells + SRBC	360	39	1650	250
2×10^5 PEC + Burro RBC + spleen cells	102	41	275	285
spleen cells + SRBC	105	0	440	0
spleen cells	35	0	25	0
spleen cells + Burro RBC	36	10	18	170

The following experiment was performed to show whether, after separating the spleen cell cultures into cells adhering to glass and those not adhering to glass, the peritoneal macrophages might be able to replace these glass-adhering cells. The cells adhering to glass were picked out of the spleen suspension by adsorption on glass beads. The remaining

free cells were pooled and incubated either with or without macrophages (Table 3). The spleen cells that did not adhere to the glass beads were capable only of a very weak immune response, 45 PFC/10^6 corresponding to only 1/10 of a normal reaction. Addition of 2×10^5 PEC to non-adhering cells led to a strong immune response against SRBC. On day 4 the yield in these cultures was 1109 PFC/10^6 cells. Addition of macrophages pre-incubated with SRBC to non-adhering spleen cells without additional antigen resulted in a clear primary response. In this test, too, production of haemolysin by spleen cells was suppressed by 2×10^6 PEC.

TABLE 3

CO-OPERATION OF NON-ADHERING (NA) SPLEEN CELLS AND PEC IN THE IMMUNE REACTION AGAINST SRBC *in vitro*

Cell combination	PFC per 10^6 harvested cells	
	day 3	day 4
spleen cells +SRBC	160	460
spleen cells	37	28
NA-spleen cells +SRBC	21	45
2×10^5 PEC +NA-spleen cells+SRBC	383	1109
2×10^5 PEC+SRBC+NA-spleen cells	480	380
2×10^6 PEC+SRBC+NA-spleen cells	0	0

DISCUSSION

The results presented permit the following conclusions:

1. Spleen cell suspensions contain a cell type which is essential for the immune response and can be replaced by peritoneal macrophages.

Phagocytosis and adherence to glass are striking properties of these cells from the spleen as well as of peritoneal macrophages. This supports the assumption that these cells could be identical. Pure macrophage samples can be obtained from the peritoneal cavity more easily than from the spleen, which is of considerable advantage for biological and biochemical investigations.

2. Production of haemolysins by 10^7 spleen cells strongly depends on the number of co-cultivated PEC. While 2×10^6 PEC suppress the reaction, 2×10^5 PEC induce a considerable increase in antibody producing cells.

A critical relationship between macrophages and immunocompetent cells has been described in new-born animals. The production of haemolysins against SRBC in new-born and 1 to 3-day-old mice could be stimulated more effectively by transfer of 5–10 million peritoneal macrophages than of 20 million (Argyris, 1968). An inhibitory effect of large amounts of macrophages on immunocompetent cells has also been described by Harris (1965) and Perkins and Makinodan (1965).

3. The production of haemolysin against SRBC in spleen cell cultures is equally well stimulated by 2×10^5 PEC preincubated either with sheep erythrocytes, with burro erythrocytes, or without any erythrocytes.

Even if only the PEC were incubated with antigen, and the co-cultivated spleen cells received none, a considerable increase of plaque-forming cells could be observed. The PEC absorbed only a small part of the SRBC antigen since no opsonizing antibodies were used. This amount of absorbed antigen was sufficient to stimulate this response. Since addition of sheep red blood cells to the combined cultures increased the number of PFC,

the macrophage-bound antigen does not seem to be sufficient for an optimal immune response. Identical behaviour of glass-adhering cells of the spleen has been described (Mosier and Coppleson, 1968). Recombined cultures of spleen cells not adhering to glass and those adhering to glass produced haemolysin against sheep erythrocytes even if only the adhering cells had contact with the antigen for 30 minutes.

The importance of macrophages for immunogenicity of antigens in the humoral primary immune response has also been described for protein antigens. Haemocyanin induces a primary response in mice much more effectively when macrophage-bound rather than in free form (Unanue and Askonas, 1968). Mitchison (1968) studied bovine serum albumin, human serum albumin, lysozyme and ovalbumin in free form as well as macrophage-bound with results identical to those of Unanue and Askonas. Perkins and Makinodan (1965) reached the opposite conclusion about the role of macrophages in the primary immune response against SRBC. From their results these authors concluded that PEC act as scavenger cells against alien erythrocytes and have no essential function in the initiation of the immune response. They believe that macrophages compete with the spleen cells for antigen. We think this conclusion is unjustified for two reasons: First, the relation of spleen cells to PEC was not optimally adjusted in these experiments. In one experiment, for example, $2{\cdot}8\times10^7$ spleen cells normally able to produce agglutinins against 10^6 SRBC in a diffusion chamber (*in vivo*-cultures in irradiated mice) were combined in this chamber with 1×10^7 PEC, which resulted in the suppression of the immune response. The number of co-cultivated PEC was in a range where an immune reaction was inhibited in our experiment. Second, the conclusion that a large number of phagocytosing cells causes an immune suppression because they compete with spleen cells for the antigen is not convincing as long as the inhibitory effect of a large number of macrophages on the transformation and the proliferation of immunocompetent cells independent of antigen is not ruled out. This latter possibility is suggested by our results. Within the first 3–4 days of cultivation we found in normal primary spleen cell cultures up to 20 per cent lymphocytes transformed into blast cells, proplasma cells and plasma cells; after addition of 2×10^5 PEC to 1×10^7 spleen cells we found up to 50 per cent transformed cells, whereas addition of 2×10^6 PEC completely inhibited the transformation of lymphocytes irrespective of the presence of sheep erythrocytes.

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