ROLE OF MACROPHAGES IN IMMUNOLOGICAL MATURATION

By BERTIE F. ARGYRIS*

(*From the Department of Zoology, Syracuse University, Syracuse, New York 13210*)

(Received for publication 13 May 1968)

Recent evidence has suggested that the initiation of antibody synthesis is a two-step process. The first step involves antigen phagocytosis and processing by macrophages (1, 2) and the second step appears to consist of the transfer of immunogenic information from the macrophage to the immunocompetent cell with subsequent initiation of antibody synthesis (3).

In most instances immunological immaturity in young or immunological deficiency in adult animals is attributed to the absence of immunocompetent cells. Recently however, it was reported by Gallily and Feldman (4) that the immunological deficiency after low doses of X-irradiation could be restored by the administration of macrophages. This suggests that the immunological insult in adult mice by low doses of X-irradiation is not directed against the immunocompetent (antibody-synthesizing) cell but is directed against the antigen-recognizing or antigen-processing cells, namely the macrophages.

In this paper we present evidence suggesting that immunological immaturity in newborn mice is not due to the lack of antibody-synthesizing (immunocompetent) cells but is due, at least in part, to the lack of antigen-recognizing or antigen-processing cells, in the form of competent macrophages. This evidence was obtained by transplanting adult macrophages into newborn mice and finding that this enhanced the rate of immunological maturation to sheep red blood cell antigens.

Materials and Methods

Adult C3H/He mice were purchased from the Jackson Memorial Laboratory, Bar Harbor, Me. Newborn mice were obtained from our own colony of C3H/He mice which were originally acquired from the Jackson Memorial Laboratory and maintained by brother and sister matings. Skin grafts can be freely exchanged between our C3H mice and the C3H/He mice obtained from Bar Harbor. All mice were housed in humidified, air-conditioned rooms. They were fed a Purina diet, fortified with occasional portions of dried milk and oats.

Treatment of Macrophage Donors—Peritoneal macrophages were provoked in adult mice by intraperitoneal injection of 3-ml sterile thioglycollate medium. The thioglycollate (Difco Laboratories, Detroit, Mich.) was prepared by adding 2.98 g powder to 100-ml cold distilled water and dissolved by heating to boiling point. After autoclaving the medium in tightly

* Supported by Grant AM-06448 from the National Institute of Arthritis and Metabolic Diseases.

capped tubes for 20 min at 15 lb. pressure, it was stored for 1 wk in the dark at room temperature. Once prepared the thioglycollate could be used for at least 2 wk if stored at 4°C.

6 or 7 days after thioglycollate injection, peritoneal cells were collected by washing the peritoneal cavity with 3 ml of Hanks' balanced salt solution (BSS) containing heparin (10 units/1 ml) and 1% inactivated calf serum. Peritoneal cells were placed in plastic centrifuge tubes and kept cold during the collection period. All steps were carried out under sterile conditions. Cells were counted in a hemacytometer using 0.5% acetic acid as a diluent. Viability was checked with the dye exclusion method, using 0.5% eosin in saline. Differential cell counts were carried out with 1% crystal violet in 0.1 M citric acid (5). Previously (5) we have reported that we can harvest 15–20 million peritoneal cells from each thioglycollate-treated mouse. The peritoneal cell population consists of 86% large macrophages, 5% medium-sized cells, 7% small lymphocytes, and 2% polymorphonuclear leukocytes and mast cells. For the sake of convenience we will, in this paper, use the terms macrophage and peritoneal cell interchangeably keeping in mind, however, that we are not dealing with a homogeneous population of peritoneal cells.

Spleen cell suspensions were prepared in sterile phosphate-buffered saline by pressing the tissue through a stainless steel sieve (6).

Treatment of Macrophage or Spleen Cell Recipients.—Young mice were injected intraperitoneally with varying doses of peritoneal macrophages or spleen cells from adult donors. Injections were carried out in 0.05–0.1 ml volumes with a 30 gauge needle and collodion applied to the injection site to prevent leakage. Only litters of seven or eight mice were used for these experiments since we found that the normal rate of immunological maturation varies considerably with litter size. Litters were divided in groups of two mice, each injected with a different dose of cells and marked by clipping the toes or tip of the tail. In some experiments the cells were rendered nonviable by heat treatment at 60°C. One group of two control mice, from each litter, was not treated with cells.

Simultaneously with the macrophage treatment, or 3 days after macrophage or spleen cell treatment, all young mice were injected intraperitoneally with 10^7 (0.05 ml of 1%), 10^8 (0.05 ml of 10%), or 10^9 (0.1 ml of 50%) sheep red blood cells (SRBC), washed three times with sterile saline. After injection the mice were returned to their mothers and each litter kept in a separate cage for the remainder of the experiment. An adult mouse, positive control for the plaque test, was injected intraperitoneally with 10^9 (0.5 ml of 10%) SRBC.

Determination of Antibody-Producing Cells.—4 days after SRBC administration the mice were sacrificed and the number of antibody-producing cells in their spleens determined with the Jerne plaque test (7). 1 day prior to the assay, 10 ml of 1.4% agar (Difco) in phosphate-buffered saline (PBS), containing 0.05% DEAE-dextran, was poured into 10-cm disposable Petri dishes. The plates were stored, upside down, in a plastic bag in the refrigerator. On the day of the assay the mice were sacrificed and a spleen cell suspension prepared from each group by pressing the tissues through a 50 mesh stainless steel screen into sterile PBS. The cells were washed once and suspended in a total volume of 0.5 ml. Cells were counted in a hemacytometer using 0.5% acetic acid. Spleen cells, together with 0.1 ml of a 20% washed SRBC suspension, were added to small tubes containing 2 ml of 0.6% Agarose (L'Industrie Biologique Francaise, Gennevilliers, Seine, France) in PBS and kept at 48°C in a water bath. A small amount of dextrose (0.4%) added to the Agarose solution, was found to increase the plating efficiency of immunized spleen cells. The contents of each small tube was mixed with a Vortex mixer and poured over the prewarmed bottom agar layer in the large Petri dishes. Three plates were prepared for each spleen cell suspension. Plate 1 received 0.2 ml of the spleen cells and plates 2 and 3 received 0.1 ml of the spleen cells. 80% of each cell suspension was therefore assayed for the presence of antibody-forming cells. Spleen cells were added to the Agarose with a 0.2 ml micropipette calibrated at 0.1 ml volume. As positive control, two plates were prepared with 2 million spleen cells, obtained from the adult immunized mouse.

All plates were incubated at 37°C for 2 hr. At this time 1.5 ml of reconstituted guinea pig complement (Gibco, lyophilized, diluted 1:10 in PBS) was added to each plate and reincubated for an additional hour. The plates were stained with benzidine (6) and stored overnight at 4°C. The next day the plaques were counted under a dissecting microscope (magnification of 7) using a grid as background. The number of antibody-forming cells was determined by calculating the number of plaque-forming cells per 1 million spleen cells. The increase in

TABLE I

Number of Plaque-Forming Cells (PFC) in Spleens of C3H Mice Treated with Adult C3H Macrophages (Mφ) and Simultaneously Sensitized with 10^8 SRBC

Age of recipients	No. of Mφ	No. litters injected	No. mice injected*		No. cells in spleen ($\times 10^6$)		No. PFC/1 $\times 10^6$ spleen cells			
			E	C	E	C	E	C	E/C	*P*
days										
	20×10^6	5	9	8	16	7	0.5	0.9	*0.6*	>0.05
1	10×10^6	4	8	6	23	8	0.5	1.1	*0.4*	>0.05
	5×10^6	4	8	6	15	8	0.6	0.7	*0.9*	>0.05
	20×10^6	4	8	8	37	26	1.0	0.7	*1.4*	>0.05
2	10×10^6	6	13	12	22	19	0.7	0.3	*2.3*	>0.05
	5×10^6	5	9	10	25	19	0.5	0.5	*1.0*	>0.05
	20×10^6	4	8	8	36	37	0.6	0.4	*1.5*	>0.05
3	10×10^6	6	13	13	38	26	2.0	0.7	*2.8*	<0.01
	5×10^6	6	11	11	48	30	0.9	0.6	*1.5*	>0.05
	20×10^6	5	10	10	51	45	1.1	2.7	*0.4*	>0.05
5	10×10^6	6	13	12	52	45	2.6	2.6	*1*	>0.05
	5×10^6	5	10	9	51	46	2.6	3.2	*0.8*	>0.05
	20×10^6	3	4	6	70	43	7.8	14.5	*0.5*	>0.05
8	10×10^6	6	12	12	63	38	8.7	10.0	*0.9*	>0.05
	5×10^6	6	11	11	52	39	10.2	12.0	*0.8*	>0.05

* E, experimental; C, control.

number of antibody-producing cells after experimental treatment was expressed as a ratio of plaque-forming cells between experimental and control groups. Statistical analysis was based on rank correlation, as described by the Mann-Whitney U test (8). The null hypothesis was rejected if *P* was smaller than 0.05.

RESULTS

Effect of Simultaneous Administration of Adult C3H Macrophages and SRBC to Young C3H Mice.—Table I records the effect of simultaneous administration of adult C3H peritoneal macrophages and 10^8 SRBC on antibody production in 1-, 2-, 3-, 5-, and 8-day-old C3H mice. 1- and 2-day-old mice fail to respond significantly to macrophage and SRBC treatment. 3-day-old mice respond to

10 million macrophages as indicated by a slight but significant increase in the number of plaque-forming cells. 20 or 5 million macrophages, injected into 3-day-old mice have no significant effect. Peritoneal macrophages injected into 5- or 8-day-old C3H mice fail to enhance antibody production significantly. Peritoneal macrophages also exert a proliferative stimulus as indicated by the increased number of cells in the spleen of macrophage-treated mice.

From these data we can conclude that there is a critical stage in development during which the lymphoid cells of young mice can benefit from the addition of adult peritoneal macrophages. Macrophage treatment before or beyond this stage is ineffective. The data also suggest that the enhancement of immunological maturation in young mice after macrophage treatment is

TABLE II

Number of Plaque-Forming Cells (PFC) in Spleens of C3H Mice Treated at Birth with Adult C3H Macrophages (Mφ) and Sensitized with 10^8 SRBC 3 Days Later

No. of Mφ	No. litters injected	No. mice injected		No. cells in spleen ($\times 10^6$)		No. PFC/1 $\times 10^6$ spleen cells			
		E	C	E	C	E	C	E/C	*P* value
20×10^6	7	13	14	42	32	1.3	0.4	*3.2*	0.036
10×10^6	7	14	16	67	37	3.5	0.5	*7.0*	0.019
5×10^6	9	18	20	50	36	2.4	0.4	*6.0*	0.01
2.5×10^6	5	10	11	51	39	0.5	0.2	*2.5*	>0.05

dose dependent. Both these points will receive further attention later in this paper.

Effect of Prior Administration of Adult C3H Macrophages on Antibody Response of Young C3H Mice to SRBC.—In the previous experiments we have shown that the spleen cells of 3-day-old C3H mice respond most favorably to macrophage treatment. In those experiments macrophages and SRBC were administered simultaneously. In order to give the transplanted macrophages a chance to adjust to their new environment before being called into action we pretreated 1-day-old C3H mice with adult C3H peritoneal macrophages and challenged these macrophage-treated mice 3 days later with SRBC. The results, recorded in Table II, illustrate a considerable increase in the number of plaque-forming cells (PFC) after transplantation of 5 or 10 million adult macrophages. It is interesting that a higher dose of macrophages, namely 20 million, is less effective than a lower dose, suggesting the importance of a critical ratio of macrophages to immunocompetent cells. A dose of 2.5 million macrophages appears subthreshold and is ineffective in stimulating antibody production significantly. In four groups of mice, macrophage treatment results in an increase in the total number of cells per spleen.

Role of Antigen Dose in Enhancement of Immunological Maturation in Young Mice by Adult Macrophages.—The previous experiment suggests that pretreatment with macrophages is more effective in stimulating antibody production to SRBC in young mice than simultaneous administration of macrophages and SRBC. We therefore adopted this routine in our further studies. In the

TABLE III

Number of Plaque-Forming Cells (PFC) in Spleens of C3H Mice Treated at Birth with 10×10^6 Adult C3H Macrophages and Sensitized 3 Days Later with 10^7, 10^8, or 10^9 SRBC

No. of SRBC	No. litters	No. mice injected		No. cells in spleen ($\times 10^6$)		No. PFC/1 $\times 10^6$ spleen cells			
		E	C	E	C	E	C	E/C	*P*
10^7	4	7	7	44	36	0.2	0.4	*0.5*	>0.05
10^8	7	14	16	67	37	3.5	0.5	*7.0*	0.019
10^9	4	8	7	39	31	3.3	0.1	*33*	0.01

TABLE IV

Number of Plaque-Forming Cells (PFC) in Spleens of C3H Mice Treated at 3 or 5 Days with Adult C3H Macrophages (Mϕ) and Sensitized with 10^8 SRBC 3 Days Later

Age of recipients	No. of Mϕ	No. litters injected	No. mice injected		No. cells in spleen ($\times 10^6$)		No. PFC/1 $\times 10^6$ spleen cells			
			E	C	E	C	E	C	E/C	*P* value
days										
3	20×10^6	6	10	11	66	44	6.2	4.2	*1.5*	>0.05
	10×10^6	7	13	13	58	40	1.7	3.7	*0.4*	>0.05
	5×10^6	7	12	13	63	44	1.6	3.2	*0.5*	>0.05
5	20×10^6	5	9	11	66	64	4.3	12.3	*0.3*	>0.05
	10×10^6	7	14	15	75	65	5.6	14.0	*0.4*	>0.05
	5×10^6	4	7	9	53	56	7.1	16.2	*0.4*	>0.05

present experiments we treated 1-day-old C3H mice with 10 million adult C3H macrophages and sensitized them 3 days later with 10^7, 10^8, or 10^9 SRBC. The results, recorded in Table III, demonstrate that an antigen dose of 10^7 SRBC is insufficient to demonstrate the enhancement of immunological maturation by macrophage treatment. Dosages of 10^8 or 10^9 SRBC are equally effective in stimulating antibody synthesis in macrophage-treated mice. The very high ratio of PFC in the group treated with 10^9 SRBC is a reflection of the low number of PFC in the control, nonmacrophage-treated group. This could be the result of immunological tolerance induced by the high dose of antigen. To

avoid any complications as a result of immunological tolerance, we selected an antigen dose of 10^8 SRBC for our further experiments.

Effect of Macrophage Treatment in 3- and 5-Day-Old Mice.—In the previous experiment we have seen that macrophage treatment of 1-day-old C3H mice, followed 3 days later by injection of SRBC, results in an enhanced antibody response. In the current experiments we have transplanted adult C3H peritoneal cells into 3- and 5-day-old C3H mice. The results, recorded in Table IV, illustrate that the immunocompetence of spleen cells from 3- or 5-day-old C3H mice is not enhanced by macrophage treatment ($P > 0.05$). The trans-

TABLE V

Number of Plaque-Forming Cells (PFC) in Spleens of C3H Mice Treated at Birth with 10 × 10^6 Heat-Killed Macrophages (Mϕ) or Spleen Cells and Sensitized 3 Days Later with 10^8 SRBC

Cell-type injected	Heat treatment	No. litters injected	No. mice injected		No. cells in spleen (× 10^6)		No. PFC/1 × 10^6 spleen cells			
			E	C	E	C	E	C	E/C	*P* value
Mϕ	60°C-1 hr	6	11	11	46	39	1.1	0.8	1.4	>0.05
	60°C-½ hr	6	15	12	45	36	0.7	0.6	1.1	>0.05
	None	10	17	19	49	39	1.7	0.6	2.8	<0.05
Spleen	60°C-1 hr	5	10	10	43	36	0.7	0.6	1.2	>0.05
	60°C-½ hr	3	8	6	35	43	1.0	0.7	1.4	>0.05
	None	6	13	12	41	40	2.4	0.7	3.4	<0.05

planted macrophages do stimulate spleen cell proliferation in the 3-day-old group of mice.

Effect of Nonviable Macrophages and Spleen Cells on Antibody Response of Neonatal C3H Mice to SRBC.—The effect of nonviable, heat-killed cells on immunological maturation in newborn mice is illustrated in Table V. 10 million macrophages or spleen cells, heated for ½ or 1 hr at 60°C are unable to increase the immune response of baby mice to SRBC. Nonheated spleen cells or macrophages in the control groups are effective. A comparison between the data in Tables II, III, and V indicates a decrease in the effectiveness of 10 million viable macrophages. In hindsight we think this is due to the occasional use of old macrophage donors in the later experiments. Preliminary results indicate that macrophages from older mice are less effective in the enhancement of immunological maturation.

DISCUSSION

The experiments described in this paper were designed to test the hypothesis that newborn mice are immunologically deficient, not because they lack func-

tional immunocompetent cells, but because they lack an antigen recognition or processing mechanism, in the form of functional macrophages. The hypothesis received experimental support by demonstrating that transplantation of adult viable peritoneal macrophages into newborn mice allows these macrophage-treated mice to respond immunologically to SRBC antigens. There appears a critical period in the development of the mouse during which the lymphoid cells can be stimulated immunologically by macrophage treatment. The lack of response before this period may be ascribed to a functional immaturity of the immunocompetent cells. The failure of macrophages to enhance antibody response beyond this critical period could be due to the fact that the mice, at this stage, have developed their own functional antigen recognition or antigen-processing mechanism and additional transplantation of macrophages has no effect.

In C3H mice, the above mentioned critical period resides around the 3rd day after birth. Enhancement of immunological maturation can be demonstrated when macrophages and SRBC are administered simultaneously to 3-day-old C3H mice, but the enhancement is more pronounced if 1-day-old C3H mice are pretreated with adult macrophages and sensitized 3 days later with SRBC. The pretreatment may give the transplanted macrophages a chance to settle and adapt to their new environment before being called up for functional duty. It should be kept in mind that the critical period during which young mice respond to macrophage treatment may vary from strain to strain. Braun and Lasky (9) have reported that macrophage treatment of 2-day-old C57BL mice results in an increased immune response to SRBC.

It should also be kept in mind that we do not claim that macrophage treatment brings the neonatal mouse up to the level of adult immunocompetence. We simply feel that the adult macrophages allow for the expression of functional activity of those immunocompetent cells which the neonatal mouse possesses. It is clearly obvious that during postnatal development the number of immunocompetent cells increases with time. A critical ratio of macrophage to immunocompetent cells at each stage during development may be important for the initiation of an immune response. This is possibly the reason why macrophage treatment of 3- or 5-day-old C3H mice fails to increase the antibody response to SRBC and why high doses (20 million) of peritoneal cells implanted into the 1-day-old C3H mouse are less effective than smaller (5 or 10 million) doses in the enhancement of immunological maturation.

In this paper we have used the terms peritoneal cell and macrophage interchangeably. The thioglycollate-induced peritoneal cells, used in these experiments, consist of 86% macrophages, 5% medium-sized mononuclear cells, 7% small lymphocytes, and 2% polymorphonuclear cells and mast cells. There is good indication that the increase in number of antibody-producing cells in neonatal mice after treatment with peritoneal cells is not due to the passive

transfer of immunocompetent cells. First of all, we have shown previously (5) that peritoneal cells from thioglycollate-treated mice fail to transfer adoptive immunity to X-irradiated recipient mice (10). Secondly, *if* the immunocompetant cells in the spleens of the neonatally treated mice were of donor origin, one would expect an increase in number when larger quantities of peritoneal cells are transferred to newborn mice. This proved not to be the case. As a matter of fact higher numbers (20 million) of peritoneal cells are *less* effective in the enhancement of immunological maturation than doses of 5 or 10 million. Subthreshold doses of macrophages (2.5 million) are also ineffective. This suggests a critical ratio of macrophage to immunocompetent cell for the successful induction of antibody synthesis. An inhibitory action of large numbers of macrophages on immunocompetent cells has also been reported by Harris (11).

Nonviable, heat-killed macrophages or spleen cells appear ineffective in enhancing immunological maturation to SRBC. This suggests that the stimulation of antibody production is dependent on the functional activity of the transplanted cells. This is also supported by our preliminary finding that 10 million macrophages from old donor mice fail to enhance antibody production to SRBC in neonatal C3H mice. Occasionally, however, we do get erratic results and find stimulation of antibody production in neonatal mice after treatment with heated macrophages. We are not sure, at the moment, whether in these instances the macrophages have not been completely killed by the heat treatment or whether during the heat treatment a stimulatory factor is released which on occasion can stimulate antibody production in young mice.

Adult viable macrophages appear to enhance antibody synthesis as well as stimulate spleen cell proliferation. We do not know whether these two phenomena are brought about by the same mechanism. We have found that spleen cell proliferation and increase in number of antibody-synthesizing cells after macrophage treatment are not always correlated. We are currently testing the effect of adjuvants on immunological maturation, in the hope of getting a better understanding of this relationship.

We are suggesting, at least for the moment, that macrophage treatment of neonatal mice supplies the young animal with an antigen recognition or antigen-processing system. It should be kept in mind, however, that this may be an over-simplification of a complex situation. There are other possible explanations for the above observed results. Only further experiments will allow us to get a better understanding of the role of macrophages in immunological maturation.

SUMMARY

1. Transplantation of peritoneal macrophages from thioglycollate-stimulated adult C3H donor mice, into 3-day-old C3H mice results in an enhanced antibody response to simultaneously injected SRBC. The increase in immuno-

competence is even more pronounced when 1-day-old C3H mice are pretreated with adult macrophages and sensitized 3 days later with SRBC. Nonviable macrophages or nonviable spleen cells are ineffective.

2. There is a critical period in the development of the neonatal mouse during which the spleen cells benefit from the addition of adult macrophages. Treatment before or beyond this stage is ineffective.

3. Very high doses (20 million) of macrophages are less effective in stimulating antibody synthesis to SRBC than doses of 5 or 10 million, suggesting that a critical ratio of macrophages to immunocompetent cells may be required for enhancing antibody synthesis in young mice.

4. The results are discussed in the light of the hypothesis that newborn mice are immunologically deficient not because they lack immunocompetent cells but because they lack an antigen recognition or antigen-processing system in the form of functional macrophages.

BIBLIOGRAPHY

1. Uhr, J. W., and G. Weissmann. 1965. Intracellular distribution and degradation of bacteriophage in mammalian tissues. *J. Immunol.* **94**:544.
2. Nossal, G. J. V., and C. M. Austin. 1966. Mechanism of induction of immunological tolerance, II. *Australian J. Exptl. Biol. Med. Sci.* **44**:327.
3. Fishman, M., R. A. Hammerstrom, and V. P. Bond. 1963. In vitro transfer of macrophage RNA to lymph node cells. *Nature.* **198**:549.
4. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in x-irradiated animals. *Immunology.* **12**:197.
5. Argyris, B. F. 1967. Role of macrophages in antibody production. Immune response to sheep red blood cells. *J. Immunol.* **99**:744.
6. Billingham, R. E., and L. Brent. 1959. Quantitative studies on tissue transplantation immunity. IV. *Proc. Roy. Soc. London, Ser. B.* **242**:439.
7. Jerne, N. K., A. A. Nordin, and C. Henry, 1963. The agar plaque technique for recognizing antibody-producing cells. *In* Cell-Bound Antibodies. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia. 109.
8. Siegel, S. 1956. Nonparametric Statistics. McGraw Hill, New York.
9. Braun, W., and L. J. Lasky. 1967. Antibody formation in newborn mice initiated through adult macrophages. *Federation Proc.* **26**:642.
10. Argyris, B. F., and B. A. Askonas. 1968. Mouse peritoneal cells: Their ability to elicit or produce antibody after exposure to antigen. *Immunology.* **14**:379.
11. Harris, G. 1965. Studies of the mechanism of antigen stimulation of DNA synthesis in rabbit spleen cultures. *Immunology.* **9**:529.