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Ontogeny of Murine Macrophages: Functions Related to Antigen Presentation

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Macrophage function in neonates was dissected into four components: antigen uptake and catabolism, cytotoxicity, antigen presentation, and the production of the lymphostimulatory molecule interleukin-1 (also called thymocyte mitogenic protein or lymphocyte-activating factor). The uptake and catabolism of ^{125}I-labeled *Listeria monocytogenes* was equivalent in macrophages from adult and neonatal mice. However, interactions between macrophages from neonates, heat-killed *Listeria* organisms, and immune T lymphocytes were impaired, and no cytocidal macrophages capable of killing tumor cells were generated. Previous studies with cells from adult mice had established that the development of cytocidal macrophages required Ia-bearing, antigen-presenting macrophages and histocompatibility at I-A between macrophages and T cells. To circumvent this requirement for antigen-presenting macrophages, an assay was used in which lymphokine was added directly to the macrophages from neonates. Strong cytocidal activity resulted. Thus, our studies confirmed that macrophages from neonates present antigen poorly but can acquire cytocidal function provided that the need for antigen-presenting function is bypassed. Similar conclusions were reached for the secretion of interleukin-1. Macrophages from neonates spontaneously secreted as much mediator as macrophages from adults, and the secretion was increased after the ingestion of heat-killed *Listeria* organisms or endotoxin. However, the marked increase in interleukin-1 production that follows antigen-macrophage-lymphocyte interaction was best seen in macrophages from adults. Macrophages from neonates could be activated to ingest C3b-coated sheep erythrocytes.

Neonates of most species are highly susceptible to infections by viruses and intracellular pathogenic bacteria and show an inability to mount an effective immune response. In the mouse, functional defects have been reported in most cells responsible for immunity (7, 9, 19, 26, 28, 31, 35–37, 40), including those of the mononuclear phagocyte lineage. Specifically, studies of macrophages from neonates have shown weak cytocidal activity against viruses (17, 32, 38), weak granuloma formation (43), and impairment of antigen-presenting function (22, 23, 27). Moreover, the impairment in immunity to virus, bacteria, and protein antigens is corrected by transplanting macrophages from adults into neonates (3, 6, 16, 17, 20, 32, 38), implying that the host macrophages are defective.

This paper examines some functions of macrophages from neonates, particularly their cytocidal activity as assayed by the killing of ^{51}Cr-labeled tumor cells and their production of the lymphostimulatory molecule interleukin-1 (1), also called thymocyte mitogenic protein (8, 39) and lymphocyte-activating factor (15). The cytocidal activity of the macrophage is triggered by proteins elaborated by T cells in response to antigen (13, 14, 30, 33). Such lymphokines, however, are released only after interaction of the T cells with antigen presented by macrophages in a process regulated by the I region of the major histocompatibility gene locus (25). An impairment of antigen presentation in the neonate could, therefore, result in the absence of cytocidal activity of the macrophage and the increased susceptibility to infection noted before (16, 17, 32, 38). It is not clear from these previous studies whether the defect in cytocidal activity of the macrophages from neonates results from a deficit of antigen presentation or from a direct inability of these macrophages to respond to lymphokines. A similar problem applies to the release of interleukin-1, inasmuch as the greatest stimulus for release of this regulatory protein follows the interaction of T cells and antigen in a step regulated by the I region of the major histocompatibility gene locus (10, 11, 44).

MATERIALS AND METHODS

Mice. Neonatal B10.A and C57BL/6 mice were raised in our colony. Adult B10.A, C57BL/6, and A/St

mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Listeria monocytogenes. Bacteria were maintained in our laboratory as described previously (21). Heat-killed bacteria were used in all in vitro experiments. Live *Listeria* organisms were used to infect mice supplying the *Listeria*-immune T cells (50% lethal dose, 2×10^5).

Lymphokine. T cell products that activate macrophages (macrophage-activating factors, or MAF) were obtained from cultures of *Listeria*-immune T cells with macrophages and heat-killed *Listeria* organisms as reported previously (12). *Listeria*-immune T cells were harvested from peritoneal exudate of mice infected 1 to 2 weeks previously with 2×10^4 live *Listeria* organisms (4, 12, 36). Macrophages were obtained from peritoneal cavities by standard procedures.

Cytolytic macrophage activity. Cytolytic activity was induced in macrophages by using one of two methods previously developed in this laboratory (12). In assay 1, peritoneal macrophages from mice of various ages (2×10^5 macrophages per 16-mm-diameter culture well) ingested heat-killed *Listeria* organisms (10^7 per well), and free *Listeria* organisms were washed away. The macrophages were then cocultured with 4×10^5 *Listeria*-immune T cells from adult mice for 24 h, after which the lymphocytes were washed away, and the cytolytic activity of the remaining adherent macrophages was assayed. The generation of macrophage cytolytic activity in assay 1 requires an initial interaction between T cells and Ia-bearing macrophages modulated by the I region of the *H-2* gene locus. In the absence of Ia-bearing macrophages or given an incompatibility at I-A between macrophages and T cells, few or no macrophages become cytolytic (12). In assay 2, macrophages were exposed to MAF or control media for 24 h, and then cytolytic activity was measured. Assay 2 explores directly the ability of macrophages to respond to lymphokine and become cytocidal independently of the H-2 haplotype of the macrophages and of the T cell elaborating the lymphokine.

In both cases, cytolytic activity was measured by the ability of macrophages to kill ^{51}Cr-labeled P815 tumor cells, after 18 h of culture, with resulting release of ^{51}Cr into the supernatant. Results are expressed in terms of cytotoxic index: cytotoxic index = 100 × [^{51}Cr (experimental supernatant) − ^{51}Cr (spontaneous release supernatant)]/[^{51}Cr (freeze-thaw supernatant) − ^{51}Cr (spontaneous release supernatant)]. The spontaneous release of ^{51}Cr varied from 20 to 36%, a value comparable to other studies employing P815 targets and an 18-h assay (2, 12).

Interleukin-1 production. The ability of supernatants of macrophage culture fluids containing interleukin 1 to induce thymocyte proliferation was tested as previously described (8, 10, 11, 39). Macrophages were cultured with immune T cells, heat-killed *Listeria* organisms, or 50 μg of *Escherichia coli* lipopolysaccharide per ml (Difco Laboratories, Westphal preparation) as in the previous section. After 24 h, the supernatants were harvested, centrifuged to remove cells and *Listeria* organisms, and cultured at various dilutions with 5×10^6 freshly harvested A/St thymocytes for 3 days. The 18-h incorporation of 1 μCi of tritium-labeled thymidine (2 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was then determined. Results are expressed as counts per minute of ^{3}H incorporation (± standard error of the mean) of the dilution of the supernatant giving optimal proliferation.

Antigen catabolism. Heat-killed *Listeria* organisms were labeled with ^{125}I (New England Nuclear Corp.) by using a lactoperoxidase technique (24). Macrophages from mice of various ages adhered to plastic 16-mm-diameter Linbro wells (2×10^5 macrophages per well) and then ingested the ^{125}I-labeled *Listeria* organisms. Free bacteria were removed by extensive washing. After various times of incubation, the supernatants were removed. Trichloroacetic acid-soluble and -precipitable fractions were assayed to determine the extent of antigen catabolism. The macrophages themselves were dissolved in Triton-X and scraped off the wells, and the trichloroacetic acid-soluble and -precipitable ^{125}I counts were assayed. Controls demonstrated no binding of ^{125}I-labeled *Listeria* organisms to the culture wells themselves.

Ingestion of IgM and IgM-C3 opsonized sheep erythrocytes. The method of Bianco et al. (5) was used to assay macrophage ingestion of immunoglobulin (Ig)M and IgM-C3 opsonized erythrocytes. Briefly, sheep erythrocytes were incubated with IgM antibody (EA19S) or with the antibody and C5-deficient A/J mouse sera (EAC). Macrophages were allowed to adhere to 10-mm-diameter glass cover slips for 1 h (2×10^5 cells per ml) at 37°C. The macrophages were then allowed to ingest EA19S or EACs. Free EA19S or EAC was washed away with 20% medium in water, which also lysed bound, but not ingested, erythro-

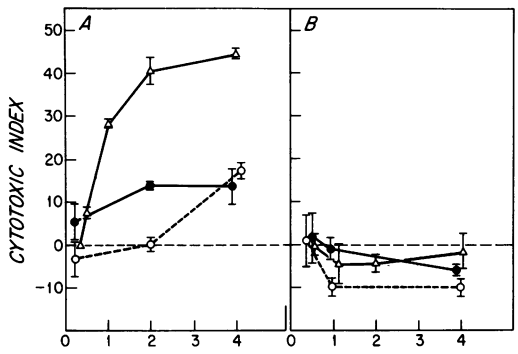

FIG. 1. Cytotoxicity by macrophages from adult versus neonatal mice after stimulation by immune T cells and heat-killed *Listeria* organisms (assay 1). (A) Peritoneal macrophages from adult (10- to 8-week-old) mice. (B) Peritoneal macrophages from neonatal (8- to 9-day-old) mice. Ordinate, cytotoxic index of ^{51}Cr release from P815 tumor target cells after correction for the spontaneous release of ^{51}Cr; abscissa, number of macrophages. △, Macrophages cultured with adult *Listeria*-immune T cells and heat-killed *Listeria* organisms. ○, Macrophages cultured with adult *Listeria*-immune T cells. ●, Macrophages cultured with heat-killed *Listeria* organisms. Spontaneous ^{51}Cr release by P815 tumor cells cultured alone or with macrophages from either adult or neonatal mice was similar (36.7%). Each point represents the mean and standard error of three measurements.

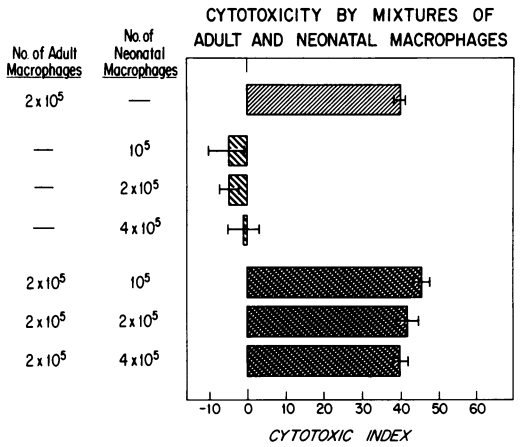

FIG. 2. Cytotoxicity by mixtures of adult and neonatal macrophages after stimulation by immune T cells and heat-killed *Listeria* organisms. Macrophages, as indicated, were cultured with adult immune T cells and heat-killed *Listeria* organisms before culture with ^{51}Cr-labeled P815 tumor cells. In control groups, macrophages not preincubated with both immune T cells and heat-killed *Listeria* organisms were not cytolytic. Spontaneous release was 36.7%. Each point is the mean and standard error of three measurements.

cytes. Ingested EA19S, EAC, and macrophages were counted with a phase-contrast microscope.

RESULTS

Cytotoxicity by macrophages from adult versus neonatal mice. Figure 1 represents four experiments and shows that, unlike macrophages from adult mice, macrophages from neonates did not become cytocidal after cultivation with adult (8- to 10-week-old) *Listeria*-immune T cells and antigen (assay 1). Figure 2 extends this observation by demonstrating that mixing macrophages from adults and neonates did not decrease the cytolytic activity of macrophages from adults. Thus, the neonatal macrophage population did not have a suppressor function that might account for their poor cytolytic activity.

Only 2 to 5% of peritoneal macrophages from neonates younger than 2 weeks were Ia positive compared with 25 to 30% of macrophages from adults (23). Thus, given the requirement for Ia-positive macrophages to present antigen to T cells in assay 1 (12), one could predict a diminished T cell activity, resulting in decreased MAF production and the lower cytolytic activity of macrophages from neonates found in Fig. 1. Our next experiments asked whether, in addition, there was also an intrinsic inability of macrophages from neonates to respond to T cell factors and become cytocidal. Figure 3 shows that macrophages from neonates become cytocidal after direct interaction with MAF (assay 2). The data in Table 1 show that the dose response of macrophages from adults and neonates to MAF was similar. Peritoneal macrophages taken from mice as young as 4 days old could be activated by MAF to kill tumor cells.

Interleukin-1 production by macrophages from adult versus neonatal mice. Macrophages from neonates and adults cultured for 24 h released equal amounts of interleukin-1. The release of mitogen was augmented in macrophages from both age groups by the uptake of heat-killed *Listeria* organisms (8, 39) or by *E. coli* endotoxin (Table 2). We showed previously that the best stimulus for increased interleukin-1 secretion is an interaction with immune T cells and antigen involving an initial antigen presentation step regulated by the I region (10, 11, 44). Table 2 shows that, after interaction with T cells and antigen, macrophages from neonates increased their secretion of mitogen, but much less than macrophages from adults did. Mixing macrophages from neonates with those from adults did not suppress interleukin-1 production after adult macrophage-T cell antigen interaction; further-

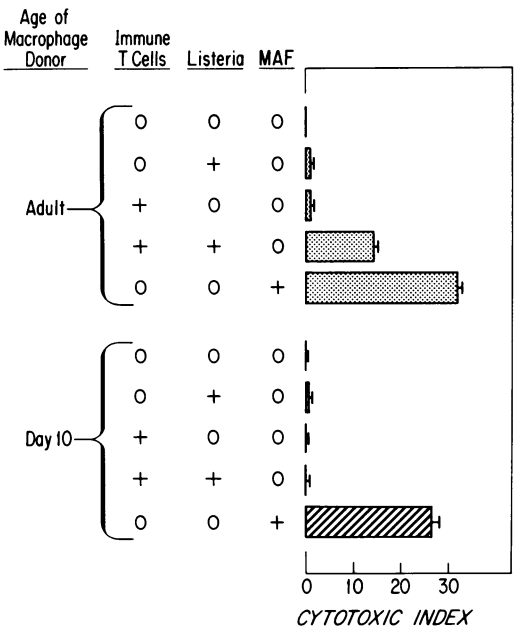

FIG. 3. Neonatal macrophages become cytocidal after stimulation by MAF (assay 2) but not after stimulation by immune T cells and antigen (assay 1). Each point represents the mean and standard error of three measurements. Macrophages from each age group were cultured as indicated and then assayed for cytotoxicity by using ^{51}Cr-labeled P815 cells. The concentration of MAF was 10% vol/vol. Spontaneous ^{51}Cr release was 20.3%.

TABLE 1. Cytotoxicity by macrophages from adults or neonates after MAF stimulation—ontogeny and dose response[a]

Expt	Dose of MAF	Cytotoxic index		Age of neonate (days)
		Adult	Neonate	
1	0	0.1 ± 0.2%	0 ± 0.1%	12
	0.5%	24.6 ± 0.4%	—[b]	
	1.0%	34.2 ± 7.5%	25.0 ± 3.8%	
	2.5%	43.3 ± 1.3%	—	
	5.0%	42.9 ± 1.1%	—	
	10.0%	45.4 ± 6.0%	43.3 ± 2.5%	
2	0	0	0.4 ± 0.2%	10
	10.0%	31.9 ± 0.7%	26.4 ± 1.4%	
3	0	0	0	10
	0.25%	0.3 ± 0.3%	0	
	0.5%	0.3 ± 0.9%	1.6, —	
	1.0%	8.7 ± 1.6%	12.9, —	
	2.5%	26.5 ± 2.3%	—	
	10.0%	26.6 ± 11.5%	25.3, —	
4	0	−1.0 ± 0.2%	−3.2 ± 3.4%	6
	10.0%	28.9 ± 0.5%	29.0 ± 1.7%	
5	0	0 ± 0.16%	—	
	10.0%	14.5 ± 1.0%	15.6, —	4

[a] Each point represents the mean and standard error of two measurements, except when the result of one measurement is given. B/6 mice were used in experiments 1 through 3; B10.A mice were used in experiment 4. Spontaneous ^{51}Cr release by P815 tumor cells alone was 20.4% in experiment 1, 20.3% in experiment 2, 23.3% in experiment 3, 27.0% in experiment 4, and 27.0% in experiment 5. In experiments 1 through 4, there were 2×10^5 macrophages per well. In experiment 5, there were 10^5 macrophages per well.

[b] —, Not done.

more, mixing supernatants of macrophages from neonates with those from adults did not suppress thymocyte proliferation (data not shown).

Antigen ingestion and catabolism by macrophages from adults and neonates. We compared the ability of macrophages from adult and neonatal mice to ingest and catabolize radiolabeled, heat-killed *Listeria* organisms (Table 3). The

TABLE 2. Thymocyte proliferation induced by supernatants of macrophages from adults or neonates[a]

Addition to macrophage cultures			Thymocyte-tritiated thymidine incorporation (cpm)			
			Age of macrophage donor			
			Expt 1		Expt 2	
T cells	*Listeria* organisms	Lipopolysaccharide	3 Mo	9 Days	7 Mo	9 Days
0	0	0	2,920 ± 22	1,274 ± 294	1,829 ± 61	1,402 ± 216
0	10^7	0	3,480 ± 757	2,811 ± 466	1,272 ± 84	1,584 ± 577
0	10^9	0	—[b]	—	3,536 ± 454	3,784 ± 50
+	0	0	1,544 ± 188	1,821, —	982 ± 177	1,542 ± 287
+	10^7	0	33,921 ± 5,441	4,854 ± 33	18,011 ± 966	4,383 ± 514
+	10^9	0	—	—	29,573 ± 28	10,988 ± 88
0	0	+	7,780 ± 589	7,047 ± 388	—	—

[a] Macrophages (2×10^5 per 16-mm tissue culture well) from adult or neonatal mice were cultured with heat-killed *Listeria* organisms, *Listeria*-immune T cells from adult mice, or 50 μg of lipopolysaccharide per ml, as indicated. After 24 h, the culture supernatants were harvested, centrifuged to remove cells or *Listeria* organisms, and assayed, using A/St thymocytes. After 3 days, the tritiated thymidine incorporation by thymocytes was measured. Background thymocyte-tritiated thymidine incorporation was 231 ± 77 cpm in experiment 1 and 731 ± 60 in experiment 2. Supernatants of immune T cells alone did not have mitogenic activity. Each point represents the mean and standard error of two measurements, except when the result of one measurement is given.

[b] —, Not done.

TABLE 3. Macrophages from adult versus neonates: uptake and catabolism of ^{125}I-labeled *Listeria* organisms

Macrophages from:	% Uptake of ^{125}I-labeled *Listeria* organisms by macrophage		Catabolism				
				% Cell associated		% Cell free	
	Expt 1	Expt 2	Time after labeling (h)	TCA[b] precipitable	TCA soluble	TCA precipitable	TCA soluble
Adult	5.9 ± 0.2	3.9 ± 0.2	0	100	0	0	0
			2.5	42 ± 5	16 ± 2	8 ± 3	34 ± 2
			5	30 ± 5	13 ± 1	3 ± 5	54 ± 2
			18	7 ± 4	8 ± 1	1 ± 10	84 ± 10
Neonate (day 10)	5.3 ± 0.1	4.4 ± 0.4	0	100	0	0	0
			2.5	40 ± 2	19 ± 1	4 ± 4	37 ± 3
			5	33 ± 11	13 ± 1	2 ± 6	52 ± 5
			18	8 ± 3	8 ± 2	3 ± 10	82 ± 7

[a] Each point represents the mean and standard error of two measurements. Cell-free counts are ^{125}I-labeled material found in the supernatant of macrophage cultures. Cell-associated counts are determined after Triton-X lysis of the macrophages. Control measurements showed that a negligible amount of ^{125}I-labeled material was adherent to the tissue culture wells in the absence of macrophages.
[b] TCA, Trichloroacetic acid.

functions were identical in the two age groups.

In Table 4, we compared the ability of macrophages from adults and neonates to become activated and ingest C3b-opsonized sheep erythrocytes (EAC3b). Bianco et al. (5) showed, using adult mice, that intraperitoneal thioglycolate or peptone injections result in stimulated macrophages that have acquired an increased ability to ingest EAC3b; after thioglycolate or peptone injection, macrophages from both age groups had an increased phagocytic activity.

DISCUSSION

Immature macrophage function contributes to the increased susceptibility of neonates to infection (16, 17, 32, 38). In this paper, we dissected the immaturity of neonatal macrophage function into antigen presentation and three different effector components: cytotoxicity, antigen uptake and catabolism, and mediator production. First, macrophages from neonates (as young as 4 days old) developed strong cytocidal activity, but only under conditions that bypassed the requirement for antigen presentation and the resulting T cell activity (Fig. 1 and 3). Neonatal macrophages had no suppressor activity in this system (Fig. 2). Second, the spontaneous and stimulated production of interleukin-1 by bacterial stimuli was comparable in macrophages from neonates and adult mice. However, macro-

TABLE 4. Both adults and neonates respond to non-immunological inflammatory stimuli

Expt	Age of macrophage donor	Stimulus	No. of macrophages per mouse	Ingestion index[a]			% Macrophages ingesting sheep erythrocytes		
				EAC3b[b]	EAC[c]	EA[b]	EAC3b[b]	EAC[c]	EA[b]
1	2 mo	None	1.7×10^6	**45.7**	0.9	1.8	**22.2**	0.9	2.7
		Thioglycolate	24.5×10^6	**233.7**	7.9	0.7	**52.5**	4.4	0.7
	13 days	None	2.6×10^5	**43.9**	13.8	—[d]	**14.9**	13.8	—
		Thioglycolate	13.5×10^5	**166.0**	9.1	0	**50.0**	11.9	0
2	4 mo	None	1.5×10^6	**21.4**	—	1	**13.0**	—	1.0
		Peptone	2.8×10^6	**81.4**	—	13.5	**55.0**	—	7.5
	10 days	None	2.1×10^5	**38.4**	—	11.7	**29.0**	—	4.2
		Peptone	1.5×10^5	**141.6**	—	41.3	**70.0**	—	25.3

[a] Ingestion index (5) = Percentage of macrophages with sheep erythrocytes after 20% Hanks balanced salt solution wash needed to lyse nonphagocytized sheep erythrocytes × average number of sheep erythrocytes ingested.
[b] EAC, EA = all rosettes made with 19S anti-sheep erythrocyte antibody.
[c] EAC = EAC made with heat-inactivated complement.
[d] —, Not done.

phages from neonates secreted much less than those from adults during their interaction with antigen and T cells (Table 2). It was expected that the secretion of interleukin-1 would not be enhanced during the interaction of neonatal macrophages with antigen and immune T cells. In this situation, secretion of interleukin-1 depends on an antigen presentation function (10, 11), which is limited in the neonate by the paucity of Ia-positive macrophages (23).

Finally, we also demonstrated that antigen ingestion and catabolism by macrophages from adults and neonates were identical (Table 3). Also, macrophages from neonates and adults had similar abilities to ingest particles (Table 4), confirming other results (41, 42). Table 4 also shows that both adults and neonates respond to non-immunological inflammatory stimuli by recruiting stimulated macrophages that had acquired an increased ability to ingest opsonized sheep erythrocytes.

In essence, the overall data indicate that the impairment of a number of macrophage functions in the neonates is due to a reduced number of Ia-positive macrophages. This results in impaired macrophage function, i.e., cytotoxicity or interleukin-1 secretion, in those situations requiring T cell activation via antigen presentation by Ia-positive macrophages. Clearly, macrophages from neonates can develop cytotoxicity, ingest and catabolize antigen, and secrete interleukin-1 in situations when T cell activation is not required. Ongoing studies in our laboratory have now indicated that the cause for the reduced expression of Ia in the neonate appears to be due to an excess production of prostaglandins (D. S. Snyder, C. Y. Lu, and E. R. Unanue, Fed. Proc., p. 1027, 1981).

The prolonged absence of Ia-bearing, antigen-presenting macrophages after birth (22, 23, 27) results in diminished T cell-dependent immunity against intracellular pathogens and thus places the newborn at increased risk from infection (16, 17, 32, 38). Thus, the delayed development of Ia-bearing accessory cells would, at first glance, seem detrimental to the preservation of the species. However, the delayed appearance of antigen-presenting macrophages may offer the animal a crucial mechanism of self-tolerance. Direct interaction between T cell and antigen has been shown in in vitro to result in antigen-specific suppressor T cell activity (18, 29). This may be analogous to the situation in the newborn and may be an important mechanism in the prevention of autoimmunity.

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