PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 431-435 (1977)

Eosinophil Response to Alum Adjuvants: Involvement of T Cells in Non-Antigen-Dependent Mechanisms[1] (39951)

RONALD S. WALLS[2]

Clinical Immunology Service, Department of Medicine, University of Cape Town, Cape Town, South Africa

Introduction. Aluminium compounds are employed experimentally and in clinical practice as adjuvants, and alum-precipitated antigens have been used to induce eosinophilia (1, 2). While investigating its adjuvant properties, aluminium hydroxide was found to attract eosinophils when given on its own. The inflammatory response to alum-precipitated antigen has been studied (3), but accumulation of eosinophils was not described. The present paper explores the capacity of alum adjuvants to attract eosinophils. It draws attention to the requirement for T cells of eosinophilia developing in the absence of antigenic stimulation. It suggests that lymphocyte activity may be stimulated directly by alum and independently of antigen recognition.

Materials and methods. Animals. CBA/Lac mice were obtained originally from the Chester Beatty Research Institute, London. Males and females aged 12 to 16 weeks were used.

Aluminium compounds and antigens. A washed commercial suspension of aluminium hydroxide (Amphogel, Wyeth Laboratories, Batch No. 2 E0305) was used. Freshly prepared suspensions (4) were used in some experiments.

Lyophilized *Ascaris* antigen (5) was reconstituted with normal saline to a final protein concentration of 0.2 mg/ml. All injections were ip unless stated otherwise.

Examination of cellular response in peritoneal cavity. Peritoneal fluid was harvested by repeated lavage with 2-ml volumes of medium to a total of 10 ml. Initially, medium 199 (Gibco) with 10% horse serum (Wellcome) and 2 units of heparin/ml, and later EDTA buffer (6), was used. Total white cells were counted and differential counts of 400 consecutive cells were made on Giemsa-stained cytocentrifuge preparations.

Intramuscular injections. Suspensions were mixed with equal volumes of trypan blue to permit localization, and 0.2 ml was injected into thigh muscles. Sections were prepared after 4 days and stained with hematoxylin and Lendrum's chromotrope.

Preparation of mice depleted of T-cells ("deprived" mice). Eight-week-old CBA/Lac mice were thymectomized, irradiated 2 weeks later from a ^{60}Co source (1000 rads), and injected intravenously with $5\text{–}10 \times 10^6$ syngeneic bone marrow cells (7). A "reconstituted" group was not thymectomized, but was irradiated and injected with bone marrow. These groups were used 50 days after irradiation. A further group of six mice underwent thymectomy without irradiation. They were used 30 days postoperatively.

Statistics. Students' *t* test was used to compare means between groups.

Results. Cellular response to aluminium hydroxide. Amphogel produced a substantial peritoneal cellular exudate containing 29.1 ± 3% (mean ± 1 SE) eosinophils on Day 6 (Table I), an increase of several hundred-fold over numbers in unstimulated mice. Mast cells accounted for 3.4 ± 0.9% of cells in resting peritoneal fluid but were not seen after injection. Increasing doses of aluminium hydroxide induced a more intense eosinophilia. A similar response was noted in DBA/2/Rij mice and in Long-Evans hooded rats.

Aluminium hydroxide- and aluminium phosphate-adsorbed tetanus toxoid (S.A. Institute for Medical Research, Johannesburg, 20 Lf/ml) injected im produced foci of necrotic muscle fibers and inflammatory cells of which eosinophils accounted for

[1] Supported by grants from South African Medical Research Council, Cancer Research Fund, Harry Crossley Foundation, and Staff Research Fund of University of Cape Town.

[2] Send reprint requests to Dr. Ronald S. Walls, Clinical Sciences Block, Repatriation General Hospital, Concord, N.S.W. 2139, Australia.

ISSN 0037-9727

TABLE I. CELLULAR RESPONSE TO ip INJECTION OF 0.2 ml OF ALUMINIUM HYDROXIDE.

Day	Number of animals	Total cells × 10^6 (mean ± 1 SE)	Eosinophils × 10^6 (mean ± 1 SE)	Neutrophils × 10^6 (mean ± 1 SE)	Mononuclear cells × 10^6 (mean ± 1 SE)
0	7	6.77 ± 0.65	0.02 ± 0.01	0.00 ± 0.00	6.33 ± 0.07
1	4	12.76 ± 3.69	0.81 ± 0.20	8.08 ± 0.20	3.66 ± 1.20
2	8	15.42 ± 2.67	1.91 ± 0.43	8.28 ± 1.30	4.65 ± 1.07
4	7	15.11 ± 3.62	3.25 ± 0.96	6.82 ± 1.68	4.59 ± 1.08
6	12	13.94 ± 1.16	3.98 ± 0.52	5.42 ± 0.64	4.84 ± 0.44
8	4	32.02 ± 5.66	9.32 ± 1.88	9.83 ± 1.63	10.16 ± 4.08
10	6	23.14 ± 6.17	4.54 ± 1.69	6.81 ± 1.56	11.37 ± 3.83
15	4	13.92 ± 4.27	1.72 ± 0.54	2.01 ± 1.01	9.62 ± 3.14
30	5	3.54 ± 0.97	0.28 ± 0.18	0.28 ± 0.18	2.64 ± 1.09

about a quarter. Bentonite or formol tetanus toxoid produced a similar inflammatory focus, but without eosinophils.

Identity of the stimulus for eosinophilia. It seems unlikely that a protein contaminant could have been responsible for eliciting eosinophilia. No nitrogen was detected in Amphogel in an Auto C, H, N analyzer, thereby excluding the presence of protein or peptide, and freshly prepared aluminium hydroxide produced a similar response. Moreover, when Amphogel was given with increasing doses of *Ascaris* antigen the response depended on the dose of aluminium hydroxide and was independent of antigen (Table II). Syngeneic serum protein precipitated with potassium aluminium sulfate induced eosinophilia, but when precipitated by heating at 56° for 12 min, it was inactive. Furthermore, only alum-adsorbed, and not formol, tetanus toxoid induced peritoneal eosinophilia (Fig. 1).

Mechanism of eosinophil response to alum. There was no evidence that alum compounds were themselves immunogenic and acting as haptens. Intraperitoneal or sc injection of aluminium hydroxide 30 days earlier did not lead to an increased peritoneal eosinophil response to a second injection. Nor did it seem likely that eosinophils were part of the peritoneal reaction to irritants. Other particulate material such as bentonite and Freund's complete adjuvant (Difco) failed to induce selective accumulation of eosinophils despite an intense inflammatory reaction (Fig. 2).

Normal, deprived, and reconstituted mice were injected with aluminium hydroxide to determine whether T cells were required for the eosinophil response (Fig. 3). Despite slightly higher eosinophil counts in resting

TABLE II. CELLULAR RESPONSE TO VARYING DOSES OF ip *Ascaris* ANTIGEN AND ALUMINIUM HYDROXIDE 4 DAYS AFTER INJECTION.[a]

Ascaris antigen (mg)	Aluminium hydroxide (ml)		
	Nil	0.02	0.2
Nil	0.01 ± 0.01 (7)	1.73 ± 0.67 (4)	3.25 ± 0.96 (7)
0.04	0.05 ± 0.02 (4)	1.30 ± 0.28 (4)	6.07 ± 1.71 (5)
0.4	0.09 ± 0.02 (5)	3.05 ± 0.64 (4)	4.08 ± 1.93 (4)

[a] Total eosinophil counts are given as mean ± 1 SE × 10^6. Numbers of animals are given in parentheses.

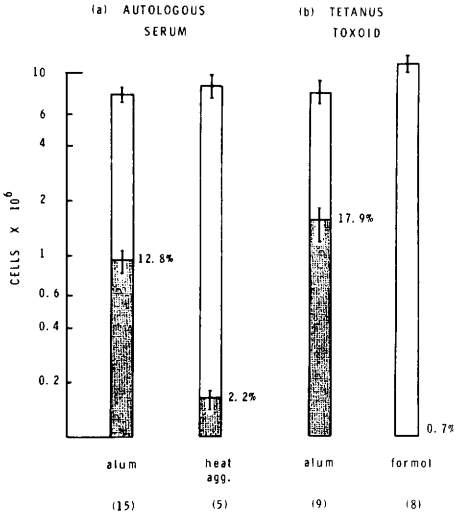

FIG. 1. Peritoneal cellular response to alum-precipitated antigens: (a) Four days after injection of 1 mg of potassium aluminium sulfate-precipitated or heat-aggregated syngeneic serum protein; and (b) six days after injection of 0.2 ml of formol or aluminium phosphate-adsorbed tetanus toxoid. Vertical lines enclose ± 1 SE. Eosinophils are also expressed as percentages of total cell counts. Figures in parentheses refer to numbers of mice in each group.

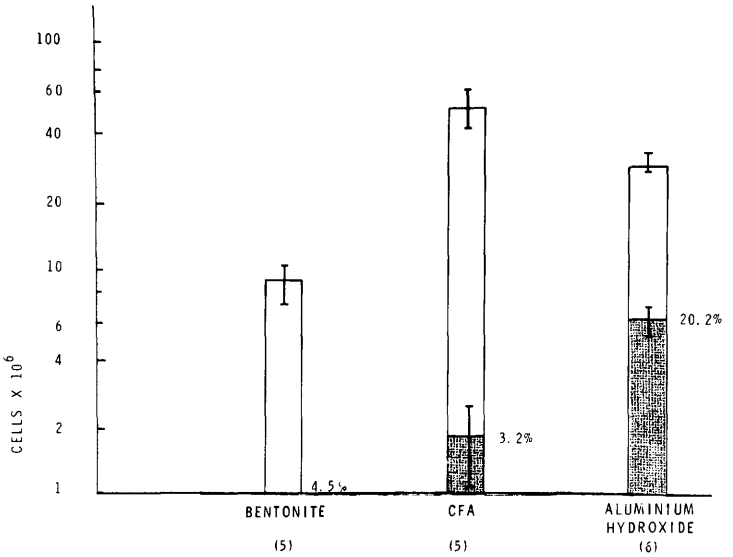

FIG. 2. Cellular responses to intraperitoneal injection of 0.1 mg of bentonite in 0.2 ml of saline, or 0.2 ml of complete Freund's adjuvant (CFA), compared with 0.2 ml of aluminium hydroxide. Peritoneal fluid was examined 4 days after injection.

peritoneal fluid, deprived mice showed a significantly diminished response when compared with reconstituted animals and with mice thymectomized but not irradiated (Table III). The ability of deprived mice to mount an inflammatory response was unimpaired as judged by total cell, macrophage, and neutrophil numbers in peritoneal fluid. Marrow production was not reduced by depletion of T cells (Table IV). Eosinophils were present in similar numbers in the three groups on Day 0. Four days after injection they were proportionately more in deprived than reconstituted mice ($P < 0.05$).

Discussion. It seems probable that eosinophils accumulated in response to aluminium compounds and not to protein antigens adsorbed to them. Induction of an "autoimmune" response to autologous protein modified by alum cannot be excluded, but it seems unlikely. Alteration of syngeneic serum proteins by heating failed to induce eosinophilia. When antigen was administered with aluminium hydroxide the response related to dose of alum and not of antigen. Furthermore, eosinophils are not prominent in autoimmune diseases.

There was no evidence that aluminium itself was immunogenic, yet mice depleted of T cells (deprived) showed a markedly diminished eosinophil response when compared with normals or controls which had been irradiated without thymectomy ("reconstituted") or which had undergone thymectomy without irradiation. This finding is in keeping with other experiments where T cells are required for eosinophilia in response to antigens (8, 9) and where T-cell involvement is specifically related to antigen recognition (10). However, this is the first demonstration that eosinophilia which is not induced by antigen also requires the presence of T cells. Several possible explanations need to be considered. First, it seems unlikely that the lack of eosinophilia in deprived mice is due to failure of the inflammatory response, as is seen after injection of antigen such as parasitic larvae (11). Depletion of T cells did not reduce numbers of macrophages and neutrophils accumulating in response to alum. Second, marrow production of eosinophils did not appear to be reduced by T-cell depletion, although kinetic studies would be required for definitive conclusions about rates of production. It seems more likely that alum had a direct effect on T cells, perhaps by causing them to secrete factors chemotactic for eosinophils (12, 13). It is also possible that alum could induce mast cell degranulation with

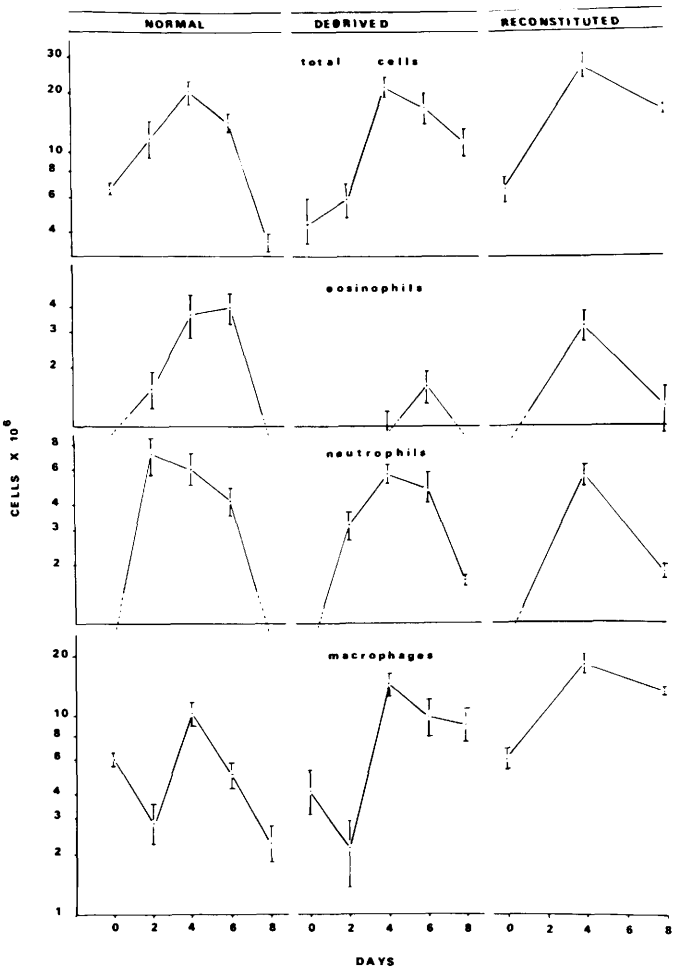

FIG. 3. Cellular response in peritoneal fluid to 0.2 ml (10 mg) of aluminium hydroxide in normal, deprived, and reconstituted mice. Vertical lines enclose ± 1 SE.

TABLE III. EOSINOPHIL RESPONSE TO 0.2 ml OF ALUMINIUM HYDROXIDE 4 DAYS AFTER ip INJECTION IN NORMAL, DEPRIVED, AND RECONSTITUTED GROUPS AND MICE THYMECTOMIZED BUT NOT IRRADIATED.[a]

Experimental group	Day 0	2	4	6	8
Normal	0.03 ± 0.01 (11)	1.55 ± 0.31 (5)	3.64 ± 0.90 (6)	3.92 ± 0.68 (8)	0.50 ± 0.20 (10)
Deprived	0.25 ± 0.17 (6)	0.47 ± 0.10 (5)	0.91 ± 0.28 (8)	1.57 ± 0.28 (8)	0.47 ± 0.15 (4)
Reconstituted	0 (4)	–	3.91 ± 0.55 (8)	–	1.23 ± 0.32 (4)
Adult thymectomy	–	–	5.25 ± 0.78 (6)	–	–
P	$>0.20^{a,b,*}$	$<0.02^{a}$	$<0.01^{a}$ $<0.005^{b}$ $<0.001^{c}$ $>0.60^{d}$ $>0.20^{e}$ $<0.05^{f}$	$<0.01^{a}$	$>0.90^{a}$ $>0.10^{b}$

[a] Total eosinophil counts are given as mean ± 1 SE × 10^6. Numbers of animals are given in parentheses.

* Comparing: (a) deprived with normal, (b) deprived with reconstituted, (c) deprived with adult thymectomized, (d) normal with reconstituted, (e) normal with adult thymectomized, (f) adult thymectomized with reconstituted.

TABLE IV. NUCLEATED CELLS IN BONE MARROW AFTER 0.2 ml OF ALUMINIUM HYDROXIDE ip.[a]

Group	Day 0		Day 4	
	Total nucleated cells $\times 10^6$	Eosinophils per 1000 nucleated cells	Total nucleated cells $\times 10^6$	Eosinophils per 1000 nucleated cells
Normal	1.93 ± 0.10	48.33 ± 6.12	1.71 ± 0.19	40.60 ± 9.60
Reconstituted	1.45 ± 0.07	51.60 ± 11.15	1.48 ± 0.10	38.20 ± 9.45
Deprived	1.90 ± 0.14	47.00 ± 4.53	1.04 ± 0.16	69.60 ± 9.69

[a] Total cells are expressed as the number counted in one femur per gram body weight. Figures represent mean ± 1 SE of five mice.

release of chemotactic factors, and that mast cell function could be defective in deprived mice.

It is desirable that investigations of the eosinophil response to antigen should include controls to test effects of adjuvant alone without antigen. These experiments suggest that accumulation of eosinophils requires the presence of T cells even in the absence of an immune response to antigen. They imply that T-cell activity *in vivo* may be initiated by events other than specific antigen recognition.

There is no definite indication that aluminium compounds are dangerous in immunization schedules in man in spite of evidence that they favor immediate hypersensitivity reactions. They attract eosinophils to injection sites, induce reaginic antibody formation (4), and prime helper T cells for IgE production in animals (15).

Summary. Aluminium hydroxide attracted eosinophils to injection sites in the absence of specific antigenic stimulation. This was markedly reduced by depletion of T cells. These observations suggest that eosinophilia caused by a nonimmunological stimulus required the presence of T cells, and imply that these lymphocytes were involved in reactions in which specific antigen recognition played no role.

1. Speirs, R. S., and Speirs, E. M., J. Immunol. **90,** 561 (1963).
2. McGarry, M. P., Speirs, R. S., Jenkins, V. K., and Trentin, J. J., J. Exp. Med. **134,** 801 (1971).
3. White, R. G., Coons, A. H., and Connolly, J. M., J. Exp. Med. **102,** 73 (1955).
4. Revoltella, R., and Ovary, Z., Immunology **17,** 45 (1969).
5. Archer, J. G., and Binet, J-L., Brit. J. Exp. Pathol. **52,** 696 (1971).
6. Paul, J., "Cell and Tissue Culture," 1st ed., p. 182. Livingstone, London (1959).
7. Davies, A. J. S., Leuchars, E., Wallis, V., and Koller, P. C., Transplantation **4,** 438 (1966).
8. Basten, A., and Beeson, P. B., J. Exp. Med. **131,** 1288 (1970).
9. Walls, R. S., Basten, A., Leuchars, E., and Davies, A. J. S., Brit. Med. J. **3,** 157 (1971).
10. Walls, R. S., S. Afr. Med. J. **50,** 1313 (1976).
11. Walls, R. S., Carter, R. L., Leuchars, E., and Davies, A. J. S., Clin. Exp. Immunol. **13,** 231 (1973).
12. Cohen, S., and Ward, P. A., J. Exp. Med. **133,** 133 (1971).
13. Colley, D. G., J. Immunol. **110,** 1419 (1973).
14. Dresser, D. W., Taub, R. N., and Krantz, A. R., Immunology **18,** 663 (1970).
15. Kishimoto, T., and Ishizaka, K., J. Immunol. **111,** 720 (1973).

Received April 25, 1977. P.S.E.B.M. 1977, Vol. 156.