Clin. exp. Immunol. (1974) **17**, 673–680.

AGGREGATION OF BUFFY-COAT LEUCOCYTES

A SIMPLE, SENSITIVE ASSAY FOR CELL-MEDIATED IMMUNITY

E. M. NICHOLLS

Chester Beatty Research Institute, Clifton Avenue, Belmont, Sutton, Surrey

(*Received* 18 *December* 1973)

SUMMARY

The addition of Tuberculin PPD to whole blood resulted in aggregation of the leucocytes. The degree of aggregation was assessed macroscopically and by two microscopic methods. The dose of PPD required to produce aggregation varied greatly between different subjects and an inverse correlation was noted between the dose of PPD needed and the Mantoux reaction.

INTRODUCTION

Several papers describing the aggregation of leucocytes in the presence of specific antigen have been published. Macrophages (Ward, Remold & David, 1970) and polymorphonuclear leucocytes (Ramseier, 1969) were attracted by leucotactic factors produced by lymphocytes from sensitized animals which had been exposed to the antigen. Following the injection of antigen into the peritoneal cavity of sensitized guinea-pigs aggregation of the peritoneal leucocytes was observed as a part of the macrophage clearance reaction (Nelson & North, 1965). Peritoneal exudate cells derived from a sensitized animal, migrating in culture chambers, were observed to aggregate if antigen was added some hours after the formation of the migratory fan of cells (David *et al.*, 1964).

In a tube method peritoneal macrophages aggregated in specifically stimulated cultures containing lymphocytes, or in cultures to which supernatants from specifically stimulated lymphocytes had been added (Gotoff & Vizral, 1972).

The aggregation of leucocytes in whole human blood has not previously been described. In this paper the phenomenon is discussed briefly and ways in which it may be used to test for sensitivity to a soluble antigen are presented.

SUBJECTS AND METHODS

Sixty-six subjects were studied. In twenty-six of these the state of sensitization to Tuberculin Purified Protein Derivative (PPD) was inferred from the medical history. Many of this

Correspondence and present address: Dr. E. M. Nicholls, School of Human Genetics, University of New South Wales, Kensington, New South Wales 2033, Australia.

TABLE 1. Sensitivity indices in sixty-six subjects, with relevant clinical information

Subject number	Age	Mantoux reaction	Number of times immunized with BCG	Relevant information	Sensitivity index
1	14	0	0	Normal	0
2	42	0	0	Normal	0
3	67	0	0	Normal	1
6	25	0	0	Normal	2
5	23	0	0	Normal	2
4	16	0	0	Normal	2
7	32	0	0	Normal	2
8	58	0	0	Normal	3
9	15	0	1	Normal	1
10	17	0	1	Normal	1
11	26	0	1	Normal	2
12	39	0	1	Normal	3
13	45	0	2	Normal	2
14	35	2 mm	0	Normal	2
15	45	2 mm	0	Normal	1
16	26	2 mm	1	Normal	2
17	29	3 mm*	3	Normal	1
18	23	3 mm	1	Normal	3
19	24	4 mm	1	Normal	2
20	33	4 mm	1	Normal	2
21	46	5 mm	2	Normal	3
22	23	6 mm	1	Normal	3
23	33	7 mm	1	Normal	2
24	26	7 mm	1	Normal	4
25	23	8 mm	1	Normal	3
26	31	9 mm	1	Normal	3
27	28	10 mm	1	Normal	4
28	29	16 mm	1	Normal	4
29	33	40 mm	1	Normal	5
30	51	n.t.	12	Normal	3
31	35	n.t.	0	Healed tuberculosis	4
32	30	n.t.	0	Healed tuberculosis	5
33	46	10 mm	2	Normal	4
34	46	Positive	0	Active tuberculosis	4
35	68	Positive	0	Active tuberculosis	4
36	82	Positive	0	Active tuberculosis	3
37	80	15 mm	0	Active tuberculosis	4
38	77	Positive	0	Active tuberculosis	4
39	37	7 mm	0	Active tuberculosis	4
40	42	12 mm	0	Active tuberculoris	5
41	54	20 mm	0	Active tuberculosis	5
42	34	16 mm	0	Active tuberculosis	4
43	24	Positive	0	Active tuberculosis	4
44	U	n.t.	0	Active tuberculosis	3
45	U	n.t.	0	Active tuberculosis	†
46	15	n.t.	3	AML	2
47	59	n.t.	3	AML	4

Table 1 cont.

48	31	n.t.	6	AML	3
49	67	n.t.	10	AML	3
50	19	n.t.	14	AML	3
51	61	n.t.	14	AML	4
52	53	n.t.	15	AML	5
53	24	n.t.	15	AML	5
45	24	n.t.	16	AML	3
55	60	n.t.	17	AML	5
56	57	n.t.	25	AML	4
57	38	n.t.	35	AML	5
58	40	n.t.	60	AML	5
59	54	n.t.	77	AML	5
60	25	n.t.	79	AML	5
61	34	n.t.	89	AML	5
62	53	n.t.	94	AML	4
63	36	n.t.	97	AML	3
64	64	n.t.	111	AML	3
65	44	n.t.	129	AML	3
66	56	n.t.	139	AML	3

N.t. = not tested. U = unknown. AML = acute myeloblastic leukaemia in full clinical remission receiving weekly BCG.

*The reaction in subject number 17 was clinically an Arthus reaction.

†No sensitivity index could be determined because of aggregation in the control.

group were part of a trial of immunotherapy as treatment for acute myeloblastic leukaemia (AML), one aspect of which was repeated immunization with Bacillus Calmette-Guérin, Glaxo (BCG), which has been described elsewhere (Powles *et al.*, 1973). All of these were in haematological remission at the time that the test was performed. Thirty subjects were tested for cutaneous sensitivity by being given an intradermal injection of 0·04 μg of Tuberculin (Evans) into the anterior aspect of the forearm. The resultant induration of the skin was measured at 72 hr at right angles to the long axis of the forearm. Twenty-one of these proved to be Mantoux-negative, i.e. they had reactions less than 6 mm in width. Ten subjects (numbers 34 to 43 of Table 1) were patients with tuberculosis and the Mantoux reactions recorded are from their hospital records.

Venous blood heparinized with 5 units/ml of preservative-free heparin (Weddel Pharmaceuticals) was used. The use of dry heparin dispersed on plastic is to be avoided for reasons given under the Results and Discussion section. To sterile plastic bijou bottles (Sterilin) 0·1 ml of saline solution of antigen (or saline alone as control) was added, followed by 0·9 ml of blood. (The antigen used in most tests was Tuberculin PPD supplied by the Ministry of Agriculture and Fisheries, Weybridge, Surrey. A weighed quantity was dissolved in saline, filtered through a 0·45 μm pore size Millipore filter, and a series of ten-fold dilutions ranging from 5000 μg/ml to 0·05 μg/ml, was prepared.) The cap of each bottle was applied tightly, the contents were mixed by shaking, and the bottles were centrifuged at 600 ***g*** for 4 min to separate the red cells, the buffy-coat leucocytes and the plasma into discrete layers. The bottles were incubated at 37°C for 20 hr. Leucocyte aggregation was assessed in three ways. (1) Aggregation of buffy-coat leucocytes was scored macroscopically (Fig. 1). (0 = 0 macroscopic clumps in whole field; + = 1–10 clumps; ++ = 11–50

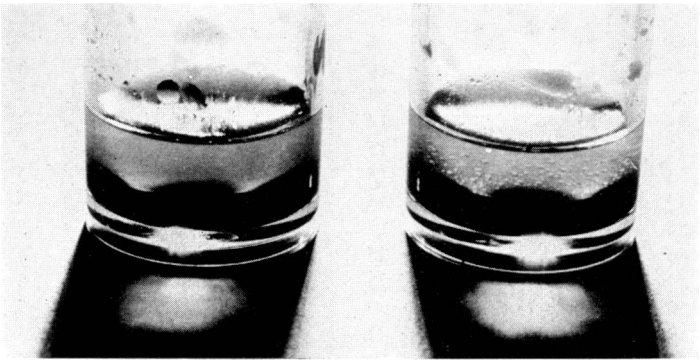

FIG. 1. Bijou bottles showing control (left-hand bottle) and positive response with aggregation of the buffy-coat (right-hand bottle).

clumps; + + + = greater than 50 clumps; + + + + = disruption of the leucocyte layer by clumping.) (2) The cap of each bottle was removed and the buffy-coat was examined en face with an incident light microscope. The aggregates were counted in an area (5 mm^2) delineated by an eyepiece graticule (Fig. 2). The bottle of blood was shaken and 0·025 ml was placed on a microscope slide with coverslip (Chance 0 gauge 22 mm^2) and leucocyte aggregates were counted in the area of the coverslip at a magnification of ×47 (Fig. 3). The aggregates were clearly visible as white spaces in a red background, the outline of cells on the upper surface of each aggregate being just visible at the magnification used. Aggregates of less than 0·06 mm in diameter were not counted, as it was found that the specific aggregation phenomenon could be differentiated by this means from the aggregation of

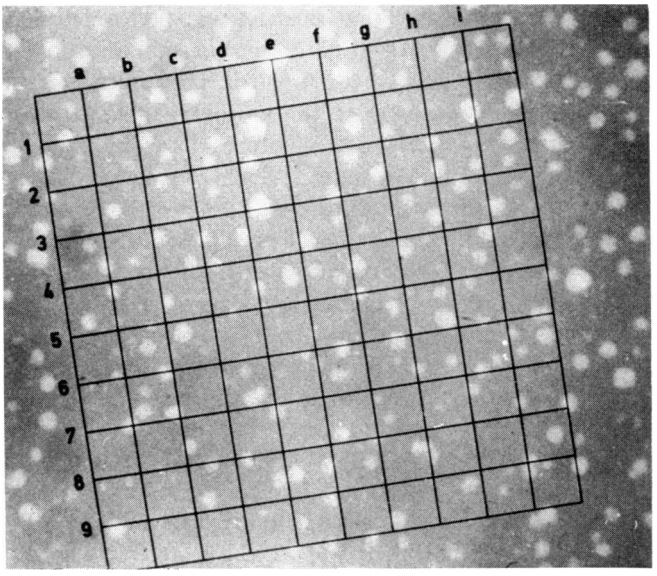

FIG. 2. Aggregates in buffy-coat, as seen through the dissecting microscope. The eyepiece graticule delineates the area in which aggregates are counted.

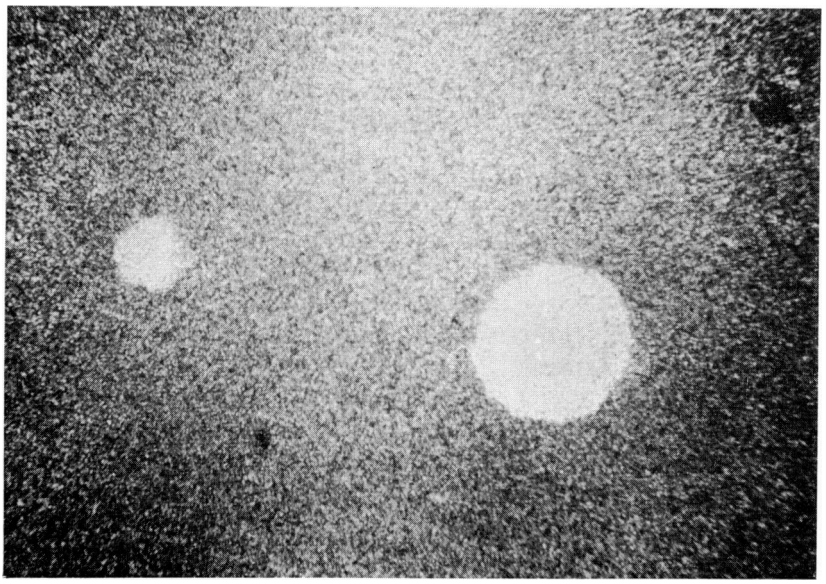

FIG. 3. Aggregates in a blood film. The aggregates stand out against the background of red cells. The faint outline of cells in the aggregate can be seen.

small numbers of leucocytes, of the order of ten or less, which occurred non-specifically.

Aggregation in a bottle for the purpose of this test was considered to be present if two or more of the following were present: a macroscopic score of + + or more, and a count of five or more for each of the microscopic methods. A sensitivity index was defined according to the dilution factor on a ten-fold dilution scale of the lowest antigen concentration which gave unambiguous clumping. The sensitivity index was the logarithm of the dilution factor for this antigen concentration, relative to the highest antigen concentration obtained in a blood–antigen mixture which was 500 μg/ml. Two examples showing the derivation of the index are given in Table 2. The Mantoux-negative subject responded to a dilution 100 times less than 500 μg/ml (sensitivity index 2) while the Mantoux-positive subject responded to a concentration 100,000 times less (sensitivity index 5).

RESULTS AND DISCUSSION

The results of testing sixty-six subjects are presented in Table 2. Sensitivity indices are presented for the thirty-three normal subjects, twelve tuberculous patients, and twenty-one AML patients. Aggregation of blood leucocytes in the presence of dilute solutions of PPD, correlated with the state of sensitization of the donor to this protein, determined either by skin test or by inference from the medical history of the subject. The greatest differences in sensitivity (as much as 100,000 times between some subjects) are found between people with no known contact with the tubercle bacillus or its proteins and no response in the Mantoux test, on the one hand, and others with known or suspected marked hypersensitivity to PPD, on the other. This extreme variation suggests that the reaction is immunologically specific and not due to a property of the tuberculin protein as such. Some experiments with the

TABLE 2. Aggregation of leucocytes in Mantoux-negative and Mantoux-positive subjects

		Concentration of antigen in test sample (μg/ml)							Sensitivity index
		0	0·005	0·05	0·5	5	50	500	
Subject number 5	Inspection of buffy-coat	+	+	+	+	++	+++	++++	2 (reaction to two ten-fold dilutions below maximum concentration)
	Count of aggregates in buffy-coat	0	0	0	1	67	108	18	
Mantoux-negative	Count of aggregates in blood film	2	2	0	1	29	43	8	
Subject number 29	Inspection of buffy-coat	0	+	++	+++	+++	+++	++++	5 (reaction to five ten-fold dilutions below maximum concentration)
	Count of aggregates in buffy-coat	0	6	16	33	48	12	1	
Mantoux-positive	Count of aggregates in blood film	0	53	77	58	47	14	2	

filtered supernatant of a preparation of *Corynebacterium parvum* (Wellcome Foundation) and with various dilutions of Varidase (Lederle, streptokinase–streptodornase) suggest that a similar response can detect sensitization to other bacterial antigens as well.

Table 2 demonstrates some aspects of the data which were used in assessing aggregation and the sensitivity index. In the majority of cases deciding which bottle first demonstrated specific aggregation was an easy matter, as it was in cases 5 and 29 (Table 2). Counts obtained by the two microscopic methods usually varied up and down in concert, but it was apparent that there were a number of variable factors determining the exact count obtained with each method.

Aggregation in the control bottle was not usually a problem and was of two types. Sometimes a large number of small aggregates of even size and distribution was found, and this type was readily distinguished from the aggregation found in bottles containing antigen. In a few cases aggregation similar in character to that found with high concentrations of antigen was observed in the control bottle. On two occasions bottles of blood heparinized using lithium–heparin dispersed on plastic were supplied for testing. In each case much non-specific aggregation occurred and it was clearly seen that most of the aggregates surrounded small particles of plastic. Defibrinated blood used on one occasion had clumps of leucocytes which appeared to have arisen around particles of fibrin or microclots of red cells. In one of the AML cases, aggregation in the control occurred at a time when there were increased numbers of cells of uncertain character, possibly monocytes or leukaemia blast cells, appearing in the circulation. In one case of active and moderately extensive tuberculosis, a conclusion as to the degree of sensitization to PPD could not be reached because of non-specific aggregation in the control. It was not possible to obtain a further sample of blood in this case.

The reproducibility of the method is demonstrated in Table 3. The test was performed many times on a few of the subjects and the sensitivity index was the same each time, or at the most varied by only one unit between the highest and lowest readings.

TABLE 3. Reproducibility of the sensitivity index on repeated testing

Subject number	Sensitivity indices obtained on repeated testing
7	1,2
21	3, 3, 3
28	4, 4
32	5, 5, 5
33	3, 4, 4, 4
34	4, 4, 4, 4
35	4, 4, 4, 4
36	2, 3, 3, 3
37	4, 4, 4, 4
38	4, 4, 4, 4
39	4, 4, 4, 4
40	5, 5, 5, 5
41	5, 5, 5, 5
42	4, 4, 4, 4, 4, 4
43	4, 4

The possible mechanisms underlying the test are being studied. The aggregates are mainly polymorphonuclear leucocytes, large mononuclear cells (monocytes), and occasional small mononuclear cells, presumably lymphocytes. A sensitized lymphocyte may become activated by antigen and by producing leucotactic factor draw to itself polymorphonuclear leucocytes and monocytes, which become firmly adherent to each other and to the lymphocyte to produce a spherical clump. An alternative hypothesis is that leucotactic/aggregation factors are secreted by the lymphocytes into the plasma and cause a gradual increase in the 'stickiness' of the white cells which then proceed to clumping irrespective of any focal effect of an activated lymphocyte.

The decline in numbers of aggregates with increasing antigen above certain levels, seen in Table 2, was studied in the leucocyte layer using the incident light microscope. Aggregation of small clumps to produce larger clumps is the first cause of a decline in numbers, while at higher concentrations of antigen, disintegration of aggregates occurs. At early stages in the reaction (as early as 1 hr after the setting up of the blood–antigen cultures) large numbers of aggregates may be seen in the bottles containing the highest concentration of antigen.

This test has simplicity and sensitivity such that it may offer the possibility of elimination of skin testing for many antigens, increase the number of antigens or antigen concentrations which can be tested simultaneously in a subject, and provide a research tool for the development of further tests for sensitization to various antigens, including those present on tumour cells. Some problems of quantitation exist, and of non-specific aggregation, but there seems to be every reason to believe that these will be overcome. The predictability of the behaviour of the leucocytes in this test, which compares more than favourably with what is found in some other tests for CMI, is probably due to the absence of drastic manipulation of the leucocytes and the retention largely unchanged of their normal milieu during the performance of the test.

ACKNOWLEDGMENTS

Many colleagues, and in particular Dr G. Currie and Professor P. Alexander, contributed to the development of this work. Special thanks are also due to Mrs C. Basham and Mr D. Glover for technical advice and to Mr K. Moreman for photography. The author was supported by a grant from the New South Wales State Cancer Council.

REFERENCES

David J.R., Al-Askari, S., Lawrence, H.S. & Thomas, L. (1964) Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93,** 264.

Gotoff, S.P. & Vizral, I.F. (1972) The macrophage aggregation assay for delayed hypersensitivity: development of the response, role of the macrophage, and the independence of humoral antibody. *Cell. Immunol.* **3,** 53.

Nelson, D.S. & North, R.J. (1965) The fate of peritoneal macrophages after the injection of antigen into guinea pigs with delayed-type hypersensitivity. *Lab. Invest.* **14,** 89.

Powles, R.L., Crowther, D., Bateman, C.J.T., Beard, M.E.J., Gauci, C.L., Wrigley, P.F.M., Naples, J.S., Fairley, G.H. & Bodley-Scott, R. (1973) Management of adult acute myelogenous leukaemia. *Brit. med. J.*, **1,** 131.

Ramseier. H. (1964) Quantitative studies on antigenic recognition. I. Immunological and nonimmunological parameters of the response. *J. exp. Med.* **103,** 1279.

Ward, P.A. Remold, H.G. & David, J.R. (1970) The production by antigen stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. *Cell. Immunol.* **1,** 162.