TESTS ON PERIPHERAL BLOOD CELLS IN MULTIPLE SCLEROSIS

by

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IMMUNOLOGICAL and epidemiological investigations have suggested that the multiple sclerosis (MS) reaction results from an abnormal response to a common childhood infection in individuals with a predisposing genetic constiution governed by the major histocompatibilitiy complex. Much effort has gone into the quest for specific infectious agents. Serological studies have pointed towards several 'membrane-associated' viruses as the most likely candidates, with measles being dominant amongst these (Fraser 1977).

The basic defect in MS could be an abnormality of cell membrane function which may not necessarily become manifest as a specific immunological defect or abnormal reactivity to specific microbial antigens. Looked at from this point of view abnormal responses to certain membrane viruses may indicate a non-specific abnormality rather than involvement of the particular virus in the disease process. Accordingly, in a study of peripheral blood cell activity in MS we have chosen to emphasize non-specific rather than antigen-specific aspects of cellular function such as blastogenic response to phytohaemagglutinin (PHA), interferon response to a virus inducer, leukocyte bactericidal and chemotactic responses and erythrocyte fragility. Because of the relatively large number of patients available for study and in view of some discordance in the literature regarding abnormality in numbers of peripheral blood cells showing markers associated with B and T lymphocytes (Sekizawa *et al,* 1974; Nowak and Wajgt, 1975; Oger *et al,* 1975; Lisak *et al,* 1975; Reddy and Goh, 1976; Lamoureux *et al,* 1976; Platz *et al,* 1976; Schauf *et al,* 1977; Nordal and Froland, 1978; Symington *et al,* 1978), we also investigated these parameters.

These studies have been carried out in a group of patients of similar racial origin living in an area where there is a high incidence of the disease (Allison & Millar, 1954).

MATERIALS AND METHODS

Patients

The 76 patients who provided specimens were aged between 17 and 68 years (mean 45 $\pm$ 11), 46 were female and 30 male. All were clinically 'probable' cases of MS as defined by Allison and Millar (1954). Most blood samples were obtained when the patients were attending out-patient clinics for routine review. Patients with severe debilitating illness were not included.

Controls

Blood specimens were obtained from normal healthy blood donors by courtesy of the Northern Ireland Blood Transfusion Service. For comparison with patient groups results from controls were selected to give an age and sex match for each patient.

Blood was collected in non-siliconized glass bottles with preservative-free heparin as anticoagulant (10 u/ml). Separation of cells from both patient and control specimens was always started within one hour of venepuncture Leucocyte preparations were obtained by layering blood on Ficoll-Triosil (Boyum, 1968) and allowing erythrocytes to sediment out at lxg for 30-40 minutes. Mononuclear cell preparations were obtained by centrifugation of blood on Ficoll-Triosil (400xg, 20 min.). Cell suspensions were washed twice with serum-free Eagle's medium (BHK Eagle's Wellcome Reagents Ltd., Beckenham).

Cell Surface Markers

E-rosette-forming cells were detected according to the method of Pang, Baguley and Wilson (1974). An E-rosette-forming cell was defined as a lymphocyte with three or more adherent erythrocytes. EAC-rosette-forming cells were detected according to the method of Luckasen *et al* (1974) using sheep erythrocytes sensitized with Wellcome rabbit anti-serum and normal mouse serum as the source of complement. Cells bearing surface membrane immunoglobulin (SmIg) were detected by direct immunofluorescence using either a polyvalent sheep anti-globulin (Wellcome), or immunoglobulin class-specific antisera obtained from Wellcome (anti-IgG, anti-IgM), Meloy (anti-IgA) or Department of Immunology, Birmingham University (anti-IgD, anti-IgE). The anti-IgA was of goat origin, the others were produced in sheep. Staining of mononuclear cells was performed at 0° for two hours followed by washing with phosphate-buffered saline (PBS) at 4°.

Serum IgA

The level of Iga in serum was determined by immunodiffusion using the 'precision' method with Hyland Immunoplates.

Lymphocyte responses to PHA

The method was based on that described by Penhale *et al* (1974) using 5 x 10^5 mononuclear cells in Eagle's medium containing 10 per cent foetal calf serum per Microtiter well. The duration of culture was 72 hours and 1 μ Ci. tritriated thymidine (5 ci. per m. mol, Amersham) was added to each well for the last six hours of culture. The same batch of PHA (PHA-P, Difco) was used for all tests and five concentrations used to give final dilutions in culture from 1/500-1/8000, three cultures being used for each dilution. Results were expressed as the mean c.p.m. in stimulated cultures minus the mean c.p.m. in unstimulated cultures.

Interferon response to Newcastle Disease Virus (NDV)

In this test 3 x 10^6 mononuclear cells were mixed with 256 haemagglutinating units of NDV (avirulent Ulster strain) in 1.5 ml balanced salt solution containing 6 per cent heat-activated human AB serum. After two hourse incubation at 37° excess virus was removed by washing the cells which were suspended in fresh medium and cultured for 22 hours at 37° in an atmosphere of 5 per cent CO_2 in air. The culture medium was then harvested, dialysed against glycine buffer (pH 3) for three days and stored at –70°. Interferon was assayed on secondary bovine kidney cells (Gresser *et al* (1974) by inhibition of encephalomyocarditis virus plaques. Titres were calculated as the dilution which inhibited 50 per cent of plaques. Equal numbers of media from control and MS cell cultures were tested in each set of interferon assays.

Leucocyte bactericidal activity

Intracellular killing of *Staphylococcus aureus* (Oxford strain) was measured by the method of van Furth and van Zwet (1973). The killing index (K_{60}) was calculated as log N_o – log N_{60} where N_o was the number of viable intracellular bacteria at the end of the initial fifteen minute phagocytosis phase and N_{60} the number of viable intracellular bacteria sixty minutes later.

Chemotaxis assay

Leucocyte chemotaxis was measured in stainless steel chambers obtained from Schleicher and Schull Inc., New Hampshire. Chambers were divided into upper and lower compartments by a Selectron filter (25 mm diameter, pore size 3 μm, type AE97). A solution of casein (5 mg/ml) in Gey's solution was injected into the lower compartment to provide a chemotactic stimulus and 1 ml of leucocyte suspension (2.5 x 10^6 cells) placed in the upper compartment. Control chambers contained Gey's solution in the lower compartment. After incubation at 37° for 30 minutes filters were removed, fixed in absolute alcohol and stained with haematoxylin (Wilkinson 1974). The chemotactic response was assessed by the leading front method (Zigmond and Hirsch 1973) and the results expressed as net migration (μm) towards the casein attractant.

Erythrocyte fragility

Thirty μl whole blood was added to 3 ml of various reagents and held at room temperature for 45 minutes. Lysis was measured by spectrophotometry of supernatants for haemoglobin. The reagents used were (a) dimethyl sulphoxide (Hopkin and Williams) 42 per cent v/v in PBS, (b) lysolecithin (Sigma) diluted in PBS (c) Phospholipase C (Lecithinase C-*CL. welchii.* Sigma) diluted in PBS and (d) hypotonic saline – various concentrations of NaCl in Sorensen's phosphate buffer.

Statistical tests

Results for all patient and control groups were compared on the basis of mean ± one standard deviation (S.D.). Student's t-test was applied to difference between the mean values of PHA responses, bactericidal activity and erythrocyte fragility to phospholipase C.

RESULTS AND DISCUSSION

Leucocyte bactericidal activity

Cells from groups of sixteen patients and controls gave mean K_{60} values of 0.6110 ± 0.2870 for the MS group and 0.9547 ± 0.3635 for the control group. This difference was not significant.

Leucocyte chemotaxis

Groups of twelve patients and controls were compared. Mean net migration towards casein was 122 ± 39 μm for the MS and 143 ± 48 μm for the control group.

Monocyte responses to PHA

The increment of ^3H thymidine incorporated in response to five concentrations of PHA shows that compared with controls MS cells were marginally less responsive to stimulation by the three lower concentrations (Table 1). Other

TABLE 1

Increase in tritiated thymidine uptake in response to different PHA concentrations. Values given as mean c.p.m. increment ± one standard deviation

	Phytohaemagglutinin Concentration				
	1/500	1/1000	1/2000	1/4000	1/8000
Controls (14)	10056 ± 7500	11612 ± 11680	11821 ± 14278	10118 ± 15458	7048 ± 13130
MS (14)	6204 ± 13147	5144 ± 10009	2716 ± 2841	1770 ± 2268	790 ± 818
Significance of difference	N.S.	N.S.	0.05>p>0.025	p>0.05	0.1>p>0.05

reports on blastogenic lymphocyte responses to PHA in MS have been conflicting (Jensen, 1968; Dau and Peterson, 1970; Davis *et al,* 1972; Offner *et al,* 1974; Knight *et al,* 1975; Lamoureux *et al,* 1976; Platz *et al,* 1976; Symington

et al, 1978). If the diminished response of MS cells to PHA is manifest only with sub-optimal concentrations of the mitogen as the present results suggest, then, since most other studies have used a single concentration, the level at which this was chosen would obviously affect the result obtained.

Monocyte interferon response to NDV

Cells from groups of twelve patients and controls were tested. There was no difference between the two groups – mean interferon titres were 1/5000 in the control group and 1/4000 in the MS group.

Monocyte membrane markers

The absolute numbers and percentages of cells demonstrating E-rosette, EAC-rosette and SmIg markers are shown in Table 2 for MS and normal groups. These tests were performed on sufficient numbers of patients to allow division

TABLE 2

Monocyte surface markers. Values given are the mean numbers of cells x 10^{-3} *per ml blood* ± *one standard deviation with the percentage of cells positive for each marker given in parenthesis*

	All Control (59)	*All MS* (59)	*'Steriod' MS* (16)	*'Non-Steroid' MS* (43)
E-Rosette	119 ± 47 (68 ± 9)	141 ± 74 (65 ± 11)	165 ± 98 (62 ± 11)	138 ± 66 (66 ± 11)
EAC-Rosette	53 ± 20 (31 ± 6)	83 ± 47 (37 ± 8)	106 ± 67 (38 ± 9)	74 ± 33 (37 ± 2)
SmIg	53 ± 59 (30 ± 7)	79 ± 47 (37 ± 13)	98 ± 66 (38 ± 14)	74 ± 39 (36 ± 13)

into a 'steroid' group (those receiving corticosteroids or corticotrophin) and a 'non-steroid' group. Although none of the differences between groups was significant it can be noted that the mean values for the 'steroid' group for all three markers showed a greater difference compared with control than those for the 'non-steroid' group. No results from patients receiving steroid therapy have been included in any other patient groups reported in this paper.

Numbers and percentages of cells showing SmIg for each immunoglobulin class are shown in Tabe 3. The only hint of difference was the slightly larger number and percentage of MS cells which reacted with the anti-IgA reagent.

TABLE 3

Number and percentages of monocytes expressing Ig class-specific markers
Values given as in Table 2

	SmIgG (31)	SmIgM (31)	SmIgA (16)	SmIgD (14)	SmIgE (14)
Controls	27 ± 15	30 ± 21	17 ± 9	41 ± 28	22 ± 17
	(15 ± 7)	(15 ± 7)	(9 ± 4)	(22 ± 7)	(11 ± 5)
'Non-Steroid' MS	37 ± 33	39 ± 30	35 ± 25	56 ± 30	22 ± 16
	(15 ± 10)	(17 ± 8)	(16 ± 11)	(27 ± 7)	(11 ± 7)

Investigations of serum IgA in MS patients gave higher mean values than controls (4.36 ± 1.88 g per 1 compared with 2.90 ± 1.71 and measurement of serum IgA in 18 specimens from each of four patients over an 18 month period showed that two out of four had levels consistently in excess of the normal mean value (i) mean 4.43 g per 1, range 3.24 – 5.75; (ii) mean 4.69, range 3.75 – 6.25) whereas the other two did not. There is therefore no consistent association of elevated serum IgA with MS.

Erythrocyte fragility

No significant differences were found between groups of 12 MS and control individuals with respect to fragility of erythrocytes in hypotonic saline, dimethyl sulphoxide, lysolecithin or phospholipase C. Caspary *et al* (1967) reported increased osmotic fragility of erythrocytes from patients with active but not with quiescent MS. Our results are in agreement with this insofar as our patients were not acutely ill with the disease.

The present observations have extended previously reported attempts to define an immunological 'profile' in MS (Lamoureux *et al*, 1976; Symington *et al*, 1978) by applying a number of non-specific tests to peripheral blood cells. A tendency to diminished function in the MS patients was suggested but no significant and characteristic abnormality was defined. We therefore conclude that either (i) there is no systemic immunological or cellular 'fault' in MS as concluded by Symington *et al* (1978), or (ii) the nature of the fault is such that presently available techniques are unable to define it, or (iii) the 'fault' may manifest itself in different ways in different patients. Although we do not have comparative data from normal individuals the observation of persistent elevation of serum IgA in some MS patients is of interest in this context.

SUMMARY

Four tests of cellular function – PHA response, interferon release, bactericidal activity and chemotactic response – all gave lower mean values for multiple sclerosis (MS) groups than for age-and-sex-matched control groups. In only one of the tests (PHA response) did the differences approach statistical significance

and then only when the lower range of PHA concentrations are used. No differences were found between MS and control groups when erythrocyte fragility was tested with hypotonic saline, dimethyl sulphoxide, lysolecithin or phospholipase C. Investigation of lymphocyte surface markers – E-rosettes, EAC-rosettes, and surface immunoglobulin (including class-specific tests) did not show any significant differences between patients and controls.

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