Experimental allergic neuritis: a new experimental approach¹

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SUMMARY The technique of macrophage migration, as a specific measure of delayed or cellular hypersensitivity, was applied to the guinea-pig model of experimental allergic neuritis (EAN) to explore the relevance of hypersensitivity to peripheral and central nervous system antigens. Results indicate that in EAN there is hypersensitivity to central as well as peripheral nervous tissue antigens. In contrast, animals with experimental allergic encephalitis showed hypersensitivity only to the central nervous system antigen used. These studies provide further evidence of the role of hypersensitivity to nervous system antigens in the pathogenesis of EAN and provide evidence of a common antigenic component to both peripheral and central nervous system antigens. A preliminary hypothesis is proposed.

Experimental allergic neuritis (EAN) is readily produced in susceptible laboratory animals by the intradermal inoculation of homogenized peripheral nerve in complete Freund's adjuvant and serves as a model for the Landry-Guillain-Barré syndrome (LGBS) of man (Waksman and Adams, 1955; Asbury, Arnason, and Adams, 1969). There is compelling evidence that EAN in animals and LGBS are similar in pathogenesis, with the small lymphocyte being the mediator of disease in both instances (Arnason, Asbury, Åström, and Adams, 1968; Behan, Lamarche, Behan, and Feldman, 1969). The clinical picture, cerebrospinal fluid (CSF) changes, and histology are similar in both. Lymphocytic infiltrates appear in the initial lesions of both EAN and LGBS with lymphocytes and lymphoblasts being found adjacent to the nerves (Åström, Webster, and Arnason, 1968).

In a separate study we have demonstrated

lymphoblasts synthesizing deoxyribonucleic acid (DNA) in the peripheral blood of animals with EAN and in patients with acute LGBS; the time course of the appearance of cells being similar in both instances. Lymphoblastic transformation was found to have a high correlation with skin testing in the primates studied. When the technique of lymphoblastic transformation was applied to patients with acute LGBS, using both central and peripheral nervous system antigens, positive transformation was obtained to *both* antigens (Behan *et al.*, 1969).

To explore the relative importance of central nervous system (CNS) and peripheral nerve antigens in the aetiology of EAN, the technique of macrophage inhibition was applied to the guinea-pig experimental model of EAN. The technique employs the principle that lymphocytes specifically sensitized to an antigen, when challenged later with that antigen, elaborate a substance—that is, macrophage inhibiting factor (MIF)—that inhibits the migration of macrophages *in vivo* and *in vitro*. The technique is a highly specific assay for the presence of delayed hypersensitivity (David, 1968).

In the application of this phenomenon, guinea-pig macrophages are packed into capillary tubes and are allowed to migrate out onto glass cover

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TABLE 1
SYMPTOMATOLOGY

Mild involvement Ataxia only noticeable on displacement. No abnormality of gait	+
Moderate involvement Ataxia present on walking. One limb or more persistently in abnormal position	++
Severe involvement Animal grossly disabled. Able to move only with difficulty	+++

Symptoms were graded by the criteria of Hall (1967).

slips in small tissue culture chambers containing test antigens.

MATERIALS

The following antigens were used in the present investigation:

PURIFIED PROTEIN DERIVATIVE (PPD) (Parke-Davis) This was used in the *in vitro* system at a concentration of 15 µg/ml.

HUMAN BRAIN CRUDE HOMOGENATE (Brain crude, (H)) This was prepared from the white matter of fresh human brain homogenized in a Ten-Broeck homogenizer. For use in tissue culture the 20% homogenate in Hank's balanced salt solution was partially clarified by centrifugation at 3,000 rpm for 30 minutes at 4° C. The resulting homogenate was diluted by the addition of tissue culture fluid '199' (TC199) with 10% guinea-pig serum to a final concentration of 0.25% vol./vol. at the time of assay. Before use the homogenate was heated to 56° C for 30 minutes.

TABLE 2

SYMPTOMATOLOGY INDUCED: GUINEA-PIG TEST GROUPS

Group A	Group B	Group C	
4	6	3	0
7	2	0	+
4	2	6	++
0	5	6	+++

The Table presents the numbers of animals in groups A, B, and C graded according to severity (see Table 1).

PERIPHERAL NERVE (PN crude(H)) Normal human sciatic nerve removed within six hours of death was stored at -70° C. At a later date the nerves were thawed, stripped of connective tissue and fat, and then autoclaved. They were then cut in 5 µ sections using a freezing microtome, then homogenized using a Ten-Broeck homogenizer. For use in tissue culture a 20% homogenate in Hank's balanced salt solutions was partially clarified by centrifugation at 3,000 rpm for 30 minutes at 4° C. A final concentration of 0.25% vol./vol. was achieved by diluting the homogenate with TC199 and 10% guinea-pig serum at the time of assay.

HUMAN MYELIN BASIC PROTEIN OF CENTRAL NERVOUS SYSTEM (BMP. CNS) These proteins of human

TABLE 3

MACROPHAGE INHIBITION TEST: NORMAL CONTROL STUDIES (12 GUINEA-PIGS)

Antigen	Concentration (µg/ml.)	% Migration
Control (no antigens)		100 ± 88
Human serum	1%	100 ± 5
PPD	15	110 ± 10
BMP. CNS (H)	10	100 ± 9
BMP. CNS (H)	15	93 ± 7
Brain (crude) (H)	1%	107 ± 6
BMP. CNS (B)	10	100 ± 4
BMP. CNS (GP)	10	120 ± 7
PN (crude) (H)	1%	129 ± 7
BMP. PN (H)	12.5	150 ± 8
BMP. PN (H)	25	117 ± 6
BMP. PN (H)	50	126 ± 6

(H) = Human. (B) = Bovine. (GP) = Guinea-pig. (M) = Monkey. PPD = Purified protein derivative of Mycobacterium tuberculosis. BMP = Basic myelin protein. PN = Peripheral nerve. CNS = Central nervous system.

(H), bovine (B), and guinea-pig (GP) origin were prepared according to the method previously described by Kies, Thompson, and Alvord (1965).

FREUND'S COMPLETE ADJUVANT (Difco) This was used as described.

METHODS

IMMUNIZATION OF GUINEA-PIG TEST GROUPS

GROUP A (EAN) Fifteen animals were immunized by subcutaneous injection into each forepaw of 0.1 ml. emulsion, containing 20 mg human sciatic nerve in 0.04 ml. Freund's complete adjuvant (Difco).

Twelve animals were used to determine macrophage inhibition.

TABLE 4

MACROPHAGE INHIBITION TEST: EXPERIMENTAL ALLERGIC ENCEPHALITIS (12 ANIMALS) (25 μ G BMP. CNS (H)/ GUINEA-PIG)

Antigen	Concentration (μ g/ml.)	% Migration
Control		100 $\pm$ 3
Human serum	1%	97 $\pm$ 5
PPD	15	10 $\pm$ 4
Brain (crude) (H)	$\frac{1}{4}$ %	62 $\pm$ 9
BMP. CNS (H)	10	47 $\pm$ 8
BMP. CNS (B)	10	65 $\pm$ 9
BMP. CNS (GP)	10	90 $\pm$ 9
PN (crude) (H)	$\frac{1}{4}$ %	113 $\pm$ 16
BMP. PN (H)	25	125 $\pm$ 9

GROUP B (EAN) Twelve animals were immunized by subcutaneous injection into each forepaw of 0.1 ml. emulsion containing 50 μ g BMP. PN (H) in 0.04 ml. Freund's complete adjuvant (Difco).

Eleven animals were used to determine macrophage inhibition.

GROUP C (EAE) Fifteen animals were immunized by subcutaneous injection into each forepaw of 0.1 ml. emulsion containing 12.5 μ g BMPCNS (H) in 0.04 ml. Freund's complete adjuvant (Difco).

Twelve animals were used to determine macrophage inhibition.

GROUP D CONTROLS Control animals were used to provide normal control studies of macrophage inhibition, and to provide matched normals for sensitized animals.

MACROPHAGE INHIBITION The method of macrophage inhibition as described by David was used

TABLE 5

MACROPHAGE INHIBITION TEST: EXPERIMENTAL ALLERGIC NEURITIS (11 GUINEA-PIGS) (100 μ G BMP. PN (H)/ANIMAL)

Antigen	Concentration (μ g/ml.)	% Migration
Control		100 $\pm$ 9
Human serum	1%	87 $\pm$ 9
PPD	15	19 $\pm$ 4
Brain (crude) (H)	$\frac{1}{4}$ %	41 $\pm$ 6
BMP. CNS (H)	10	25 $\pm$ 3
BMP. CNS (B)	10	34 $\pm$ 6
BMP. CNS (GP)	10	95 $\pm$ 2
PN (crude) (H)	$\frac{1}{4}$ %	41 $\pm$ 5
BMP. PN (H)	25	29 $\pm$ 6

with minor modifications. Macrophages were harvested from peritoneal exudates three days after the intraperitoneal injection of 30 ml. sterile bland mineral oil (Philip Roxane laboratories). Paracentesis was carried out using 150 ml. Hank's balanced salt solution for lavage.

After separation, the aqueous cell-containing phase was centrifuged at 1,200 rpm at 4° C for 12 minutes. Cells were washed by resuspension in 15 ml. Hank's balanced salt solution three times successively. Microscopically, these cells averaged 95% oil laden macrophages and 5% lymphocytes. After final washing, the cells were suspended in TC199 and made up to a 10% suspension. Fifty lambda non-heparin-

TABLE 6

MACROPHAGE INHIBITION TEST: EXPERIMENTAL ALLERGIC NEURITIS (12 GUINEA-PIGS) (40 μ G CRUDE HUMAN NERVE/ANIMAL)

Antigen	Concentration (μ g/ml.)	% Migration
Control		100 $\pm$ 5
PPD	15	24 $\pm$ 3
Brain (crude) (H)	$\frac{1}{4}$ %	37 $\pm$ 5
BMP. CNS (H)	10	34 $\pm$ 4
BMP. CNS (B)	10	37 $\pm$ 8
BMP. CNS (GP)	10	90 $\pm$ 8
PN (crude) (H)	$\frac{1}{4}$ %	33 $\pm$ 8
BMP. PN (H)	25	42 $\pm$ 11

ized capillary tubes were filled with the cell suspension and sealed with paraffin at one end and centrifuged at 600 rpm for five minutes at ambient temperature. The tubes were then cut at the cell fluid interphase and the cell-containing portion, sealed at one end, placed in a Mackenness type tissue culture chamber (David, 1968b). Normal autologous guinea-pig serum (10%) was used to supplement the media containing the test antigen. Antibiotics were not used. A minimum of four chambers (eight capillary tubes) were used for each antigen tested in each experiment.

The chambers were incubated at 37° C and subsequently read at 18 and 24 hours. Areas of cell migration were determined by projection and planimetry using a microscope filled with a Zeiss projecting prism.

RESULTS

Clinical evaluation for EAN was carried out using the criteria of Hall (1967) (Table 1).

FIG. 1. Microscopic section: guinea-pig sciatic nerve stained with luxol fast blue ($\times 400$). There is demyelination and scattered mononuclear infiltrates in a lesion from an animal immunized with 40 mg of human sciatic nerve in Freund's complete adjuvant; and killed at 21 days.

Approximately two to three days before the onset of any 'neurological symptoms' the animals of all three test groups were noted to be less active in their cages when compared with normals. Fur about the hind quarters began to be stained with urine at this time. Mild symptoms appeared in the animals in group A between the 12th to 18th day then began to subside in two to three days. Animals were reluctant to move and those who progressed very shortly demonstrated

gait-abnormality. They tended to have one or both hind limbs in an extended position. In group B, approximately 14 to 21 days after sensitization, the animals displayed decreased activity, and variably showed some urine staining of their fur. There was no dramatic change in these animals until about the 30th day after sensitization. Two animals died suddenly after a few hours of illness. The results are summarized in Table 2.

FIG. 2. Microscopic section: lesion in guinea-pig spinal cord, stained with haematoxylin and eosin ($\times 400$), demonstrating perivenular mononuclear infiltration in an animal immunized with 25 μ g encephalitogenic protein (human), in Freund's complete adjuvant and killed at 21 days.

FIG. 3. Microscopic section: guinea-pig sciatic nerve stained with haematoxylin and eosin ($\times 100$). The specimen from an animal immunized with 100 μ g of a pH_3 extract of human sciatic nerve (BMP. PN (H)) and in Freund's complete adjuvant killed at 21 days. The section demonstrates Schwann cell proliferation.

The results of macrophage inhibition are summarized in Tables 3 to 6. Cells from animals sensitized to sciatic nerve, or to pH_3 extracts of sciatic nerve demonstrated inhibition of macrophage migration when tested with either antigen *in vitro* and also to CNS basic myelin protein. In contrast, cells from animals sensitized to CNS basic myelin protein exhibited inhibition of macrophage migration only in response to CNS and basic myelin protein.

HISTOLOGY All animals sensitized with human sciatic nerve showed lesions typical of EAN in their sciatic nerves (Fig. 1). Animals of group B showed changes of a lesser order that are the subject of further study. The changes are much less striking and do not represent typical changes of EAN (Fig. 2). Animals sensitized with human CNS basic myelin protein demonstrated typical changes of EAE in their central nervous system white matter (Fig. 3). No similar changes were seen in any control animals.

DISCUSSION

The present study, using the highly specific technique of macrophage inhibition demonstrates that delayed hypersensitivity to a specific antigen is a factor in EAN. In the guinea-pig with EAN there is specific delayed hypersensitivity to the antigenic components of crude human sciatic nerve, and to those in pH_3 extracts of such nerve. This does not develop in guinea-pigs sensitized with pH_3 extracts of human brain white matter—that is, basic myelin protein of human central nervous system. Animals with EAN, in contrast, showed cross-sensitivity to central nervous system antigens both crude and purified.

The macrophage inhibition test is now well established as a reliable method for the detection of delayed hypersensitivity *in vitro* (David, 1968a, b). It has been shown that this technique is extremely sensitive in recognizing specific antigens (David and Schlossman, 1968). The technique therefore lends itself to the detection of delayed hypersensitivity in EAE and EAN, two experimental diseases considered to be mediated by this mechanism. Previous experiments using this method have shown positive inhibition of macrophage migration by lympho-

cytes from guinea-pig and rats with EAE, using either crude brain or encephalitogenic protein as test antigen (David and Paterson, 1965; Brockman, Stiffey, and Tessar, 1968; Hughes and Field, 1968; Behan, Sheremata, Lamarche, Lisak, and Kies, 1970).

The theory that EAN is a disease mediated by delayed hypersensitivity is supported by several observations. Histologically, lymphocytes can be demonstrated throughout the affected nerves; and lymphoblasts and dividing lymphocytes have been found adjacent to EAN lesions in rats (Aström *et al.*, 1968). Perhaps the strongest evidence implicating cellular mechanisms to date is the ability of passively transferred lymph node cells from rabbits with EAN to produce lesions of EAN in recipient animals and the failure of serum to do so (Winkler, 1965). Cutaneous hypersensitivity to testing with peripheral nerve has been recorded (Waksman and Adams, 1965).

The antigen involved in EAN is unknown. The ' pH_3 extract' of sciatic nerve cannot be considered a pure antigen. The production of the clinical features associated with EAN and the histological lesions, albeit atypical, seen in our animals' nerves when immunized with the ' pH_3 extract', suggests that the antigen responsible for the disease may be contained in this particular fraction. The exact elucidation awaits further experimentation.

In other studies using the technique of macrophage inhibition, delayed hypersensitivity to human peripheral nerve homogenates was found in patients with LGBS syndrome (Rocklin, Sheremata, Feldman, Kies, and David, 1971; Sheremata, Rocklin, Feldman, Kies, and David, 1972). This finding adds further evidence to the theory that both EAN in animals and LGBS in man have a common immunopathogenesis. This study, therefore, adds further support to the concept that delayed hypersensitivity to peripheral nerve antigen or antigens is involved in EAN. The nature of this antigen has not yet been clearly elucidated.

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