Immune responses in experimental allergic neuritis

R A C HUGHES,* M KADLUBOWSKI,* I A GRAY,* AND S LEIBOWITZ†

From the Department of Medicine* and the Department of Pathology,†
Guy's Hospital Medical School, London

SUMMARY The antibody and cell mediated immune responses were investigated in inbred Lewis rats with experimental allergic neuritis (EAN) induced by either P₂, a protein purified from the bovine cauda equina nerve roots, or whole bovine nerve root myelin. In the P₂ immunised animals both antibodies to P₂ detected by radioimmunoassay and cell-mediated immunity to P₂ assayed by skin testing appeared before the onset of EAN and persisted during and after the disease. In the myelin immunised animals the antibody titres were lower and somewhat delayed and the skin tests became negative at the height of the disease. Complement-fixing antibodies to galactocerebroside, which have been implicated in the production of demyelination under some circumstances, could not be detected in the serum after immunisation with either P₂ or myelin. EAN was transferred passively with lymph node cells from rats immunised with either P₂ or myelin although anti-P₂ antibodies could not be detected in the serum of recipients with EAN. The results favour a cell-mediated immune response to P₂ as the most important pathogenetic mechanism in EAN induced with whole myelin in the rat.

Experimental allergic neuritis (EAN) produced by immunisation with an emulsion of peripheral nerve and adjuvant is an important model of human acute inflammatory polyneuropathy (Guillain-Barré-Strohl syndrome) which it closely resembles histologically. Study of its immunological mechanisms was delayed because in most species the disease was contaminated by inflammation of the central nervous system, that is experimental allergic encephalomyelitis (EAE), and the identity of the neuritogenic antigen was not known. It has recently been shown that bovine peripheral nerve myelin injected into inbred Lewis rats produces pure EAN without EAE,^{1,2,3} and that P₂ basic protein purified from that myelin is responsible for the neuritogenic activity.^{1,4,5} This paper describes the humoral and cellular responses to P₂ during EAN and the passive transfer of the disease with lymph node cells.

Methods

Male inbred Lewis rats were injected as previously described^{1,4} with 0.25 mg bovine P₂ or 2.5 mg bovine

peripheral nerve myelin, emulsified with adjuvant containing 0.5 mg *Mycobacterium tuberculosis*, and killed after 7-42 days. The severity of neurological signs was graded 0=normal, +=limp tail, ++=paraparesis, +++=paraplegia, tetraparesis or death. The mean scores in table 1 refer to the signs of 4-6 rats at the time of sacrifice when blood and lymph nodes were collected. The scores correspond closely to those obtained in previous experiments with 10 rats immunised with the same dose of P₂ and 10 with the same dose of myelin. Histological examination was

Table 1 Percentage ¹²⁵I-P₂ binding of sera from P₂ and myelin immunised rats in relation to neurological signs

Days after immunisation	P ₂ immunised		Myelin immunised	
	Neurological score	¹²⁵ I-P ₂ binding	Neurological score	¹²⁵ I-P ₂ binding
0	0	3.5±0.5	0	3.5±0.5
7	0	5.3±1.5	0	5.2±3.2
9	0	27.4±7.5	—	not done
10	0	30.7 (30.1, 31.3)*	0	6.4±2.3
12	0.25±0.5	45.9 (44.4, 47.4)*	2.3±0.6	10.7±3.1
14	1.4±0.9	60.3±7.2	2.8±0.4	20.3±20.5
21	1.75±0.5	74.9±4.6	1.3±1.1	77.0±2.3
42	0	68.0 (57.5, 78.9)*	0.5±0.7	77.5±2.6

Mean ± SD of 3-6 rats except for values marked * which are means of the two bracketed observations.

Address for reprint request: Dr RAC Hughes, Department of Neurology, Guy's Hospital, London SE1 9RT, England.

Accepted 22 May 1981

performed on paraffin embedded sections of the sciatic nerves and cauda equina stained with haematoxylin and eosin or luxol fast blue. Antibodies to P_2 were detected by radioiodinating P_2 according to the method of Sarvas *et al.*,⁶ incubating 2 ng of this $^{125}\text{I}-P_2$ with 50 μl appropriately diluted test serum in a volume of 500 μl overnight, and finally precipitating the bound P_2 with 10% polyethylene glycol. The radioactivity of the precipitate was counted and expressed as a percentage of the total counts added. Skin tests were performed by injecting 50 μg P_2 or purified protein derivative of tuberculin (PPD) in 0.1 ml phosphate buffered saline intradermally into the flank of the rats under ether anaesthesia. The depth of induration was assessed 24 hours later by measuring the thickness of a fold of skin containing the skin test with calipers and subtracting the thickness of the skin fold on two sides.

Lymph node cell transformation was tested by a modification of our technique for human peripheral blood mononuclear cells.⁷ The rats were exsanguinated under ether anaesthesia and the draining axillary lymph nodes were dissected, teased in tissue culture medium, and sieved through a fine stainless steel mesh (0.84 mm). The lymph node cells were washed twice and cultured in the wells of a microtitre tray at 400,000 viable cells per well in Minimum Essential Medium modified with Earle's Salts (Flow Lab 12-102-49) containing hepes 20 mM, 10% human AB serum, glutamine 200 mM, penicillin and streptomycin. The required amounts of antigen were added and the cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C. The cells were harvested after 72 hours, 20 hours after the addition of 0.8 μCi ^3H -thymidine.

In the lymph node cell transfer experiments the antigen emulsion for the donors was usually distributed between all four feet. All the lymph nodes were harvested after 9 or 21 days and then teased and sieved to prepare a single cell suspension. The cells were washed twice and then injected into the dorsal penile vein in a volume of 1–2 ml. The recipients were observed daily and killed after 14 days. Antibodies to galactocerebroside were sought with a micro-complement fixation test with 5 μg galactocerebroside, and cholesterol and lecithin as auxiliary lipids.

Results

The neurological signs produced by whole peripheral nerve myelin were similar but more severe than those produced by P_2 (table 1). After 11–12 days the rats developed limp tails, then hind limb paresis and sometimes paraplegia, tetraparesis or death. With the doses used all the rats became affected after 14 days. The histological appearances of cellular infiltration and demyelination, assessed on paraffin fixed material stained with haematoxylin and eosin or luxol fast blue, were

similar in rats immunised with either purified P_2 protein or whole myelin.

After immunisation with P_2 increased $^{125}\text{I}-P_2$ binding was detected in the serum at nine days, rose until 21 days and remained high at 42 days (table 1). After immunisation with whole myelin the $^{125}\text{I}-P_2$ binding rose more slowly, but by 21 days achieved a level similar to that after P_2 immunisation. Rats immunised with adjuvant alone did not produce significant $^{125}\text{I}-P_2$ binding (the mean percentage binding of 11 samples was 3.8 ± 0.7). Complement fixing antibodies to galactocerebroside were not detected in any of the sera although rabbit antigalactocerebroside antiserum reacted strongly in the same test.

In 21 complete adjuvant immunised control rats injection of P_2 produced no erythema or induration (mean depth of induration $\pm\text{SD} = 0.00 \pm 0.04$ mm). In the P_2 and myelin immunised animals P_2 produced positive skin tests 7–10 days after immunisation (table 2). The induration was less than with similar tests with PPD (table 3) and erythema, although present, was difficult to measure accurately. In the P_2 immunised rats the positive skin tests persisted during the disease whereas in the myelin immunised rats the skin reaction to P_2 and PPD diminished (tables 2 and 3).

In the lymph node cell transformation tests, addition of P_2 100 $\mu\text{g}/\mu\text{l}$ produced an increment in ^3H -thymidine incorporation of lymph node cells from all the groups of rats. Only the cells from the rats immunised with pure P_2 had a significantly

Table 2 Skin tests with 50 μg P_2 in P_2 and myelin immunised rats

Days after immunisation	P_2 immunised rats	Myelin immunised rats
7	0.13 $\pm$ 0.1	0.22 $\pm$ 0.08
10	0.47 $\pm$ 0.28	0.27 $\pm$ 0.16
12	0.43 $\pm$ 0.06	0.37 $\pm$ 0.16
14	0.43 $\pm$ 0.15	0.125 $\pm$ 0.12
21	0.30 $\pm$ 0.10	0.02 $\pm$ 0.03
42	0.40 $\pm$ 0.11	0.02 $\pm$ 0.14

Mean induration (mm) $\pm$ SD for 4 rats.

Table 3 Skin tests with 50 μg PPD in adjuvant, P_2 and myelin immunised rats

Days after immunisation	Adjuvant immunised rats	P_2 immunised rats	Myelin immunised rats
0	—0.05*	—0.05*	—0.05*
7	0.61 $\pm$ 0.15	0.31 $\pm$ 0.14	0.23 $\pm$ 0.19
10	1.08 $\pm$ 0.09	1.02 $\pm$ 0.21	0.68 $\pm$ 0.40
12	1.04 $\pm$ 0.21	1.00 $\pm$ 0.44	1.30 $\pm$ 0.36
14	1.76 $\pm$ 0.67	1.56 $\pm$ 0.55	0.18 $\pm$ 0.10
21	0.93 $\pm$ 0.11	0.38 $\pm$ 0.18	Not done
42	0.50 $\pm$ 0.10	0.49 $\pm$ 0.23	0.20*

Mean induration (mm) $\pm$ SD for 4 rats.

*One observation.

larger increment than those from normal rats (table 4). P_2 20 μ g/ml caused increased transformation only of the cells from rats immunised with P_2 .

Six animals received a single intraperitoneal injection of 9–12 ml of pooled antimyelin serum. Four of these were given serum from rats immunised 21 days earlier with 2.5 mg bovine myelin; one received 14 day and another 9 day serum. No neurological signs were observed during the next two weeks and no histological evidence of demyelination or inflammation was found in the sciatic nerves or cauda equina. By contrast mild neurological signs and mild or moderate cellular infiltration of the sciatic nerves and cauda equina were consistently observed following transfer of 2.0×10^8 or more lymph node cells from donors sensitised nine days earlier with P_2 or myelin (table 5). Thymocytes and spleen cells from the same donors were ineffective. The effect was lost if the cells were killed by heating to 56° for 30 minutes. Lymph node cells from donors sensitised 21 days earlier did not transfer the disease nor did lymph node cells from donors immunised with adjuvant alone. The ^{125}I - P_2 binding of the sera of the two rats with passively transferred

EAN in experiment 6 was 2.7% and 4.1%, not significantly different from that of normal rats (table 1). On the other hand the serum of the recipient of 21 day cells in experiment 9 bound 8.9% indicating the presence of circulating antibody although no neurological signs or histological lesions of EAN were detected.

Discussion

Immunisation of Lewis rats with either whole bovine intradural root myelin or purified P_2 stimulates production of antibodies to P_2 which are readily detected by our ^{125}I - P_2 binding assay. The antibody level does not correspond closely to the course of the neurological signs of EAN (table 1). For instance in the P_2 immunised animals although circulating antibody was clearly present on day nine, one or two days before the onset of neurological signs, peak levels were not achieved until after 21 days by which time surviving animals had either recovered or were improving. The antibodies remained at a high level at 42 days when all the rats had recovered. In the myelin immunised animals the discrepancy between antibody levels and appearance of neurological signs was even more obvious since the antibody levels remained relatively low at 12 and 14 days, although these rats were more severely affected than those immunised with P_2 . The method used investigated P_2 bound by all classes of antibody so that the present experiments do not eliminate the possibility that minor subclasses of immunoglobulin or tissue bound antibody play a part.

The absence of complement-fixing antibodies to galactocerebroside from the serum of rats with EAN makes it unlikely that these play a part in the pathogenesis of EAN under the conditions of these experiments. Under other circumstances antibodies to galactocerebroside are important in

Table 4 Lymph node cell transformation in the presence of P_2 14 days after immunisation

Immunisation	No of rats	Background incorporation without antigen	Increment with P_2 100 μ g/ml	Increment with P_2 20 μ g/ml
None	10	280 $\pm$ 310	530 $\pm$ 670	90 $\pm$ 140
Adjuvant alone	9	300 $\pm$ 330	810 $\pm$ 1000	88 $\pm$ 100
P_2	8	270 $\pm$ 150	2790 $\pm$ 1250*	450 $\pm$ 210*
Myelin	3	470 $\pm$ 190	850 $\pm$ 370	40 $\pm$ 190

*H-thymidine incorporation per 400,000 cells. Mean $\pm$ SD, DPM.

*The increment with P_2 of the cells from P_2 immunised rats is greater than that of cells from the normal rats ($p < 0.01$, two-tailed Wilcoxon rank sum test) or of the cells from the adjuvant immunised rats ($p < 0.01$).

Table 5 Induction of EAN by passive transfer of cells

Experiment	Cell type	Immunogen	Day of transfer	No of cells transferred $\times 10^8$	Neurological signs		Histological disease	
					Incidence	Score	Incidence	Score
1	LNC*	myelin	9	2.8	3/4	0.75	4/4	2.0
2	LNC	myelin	9	2.0	0/3	0	2/3	0.75
3	LNC				2/2	1.0	2/2	2.5
	thymus	P_2	9	2.0	0/2	0	0/2	0
	spleen				0/2	0	0/2	0
4	LNC	myelin	9	2.0	2/3	0.67	3/3	1.67
5	LNC	P_2	9	4.0	2/2	2.0	2/2	2.0
	LNC			4.0†	0/2	0	0/2	0
6	LNC	P_2	9	2.5	2/2	1.5	2/2	2.0
7	LNC	myelin	21	2.0	0/2	0	0/2	0
8	LNC	P_2	21	2.5	0/1	0	0/1	0
9	LNC	myelin	21	2.0	0/1	0	0/1	0

LNC = lymph node cells.

† = cells killed by heating at 56° for 30 minutes before injection.

immune mediated demyelination since rabbits repeatedly immunised with galactocerebroside develop demyelination and macrophage infiltration,⁹ which can be reproduced by intraneural injections of antigalactocerebroside serum.¹⁰ The myelin splitting preceding cellular infiltration and prominent Schwann cell death in the galactocerebroside induced lesion are not described in EAN induced by whole nerve or myelin¹¹ nor have we noted them in our own material. Hoffman *et al*³ were unable to produce disease in rats with galactocerebroside so that species differences are probably important.

The time course of skin test responses to P₂ did not correspond to the development and disappearance of neurological signs any more closely than the antibody levels (table 2). In the P₂ immunised rats the skin test became positive after 10 days just before the onset of neurological signs and remained positive for the next three weeks. In the myelin immunised rats a positive skin test reaction with P₂ appeared at 7–12 days but then disappeared despite the fact that these animals had disease which was at least as severe as those immunised with P₂. The skin test reaction with PPD was depressed in the P₂ immunised rats after 21 days and the myelin immunised rats after 14 days compared with the adjuvant immunised controls. It is possible that the disappearance of the positive skin test to P₂ in the myelin immunised animals and depression of PPD response in both groups represents a non-specific response to stress. Another possible mechanism would be that the available sensitised cells have left the circulation having been attracted by the target antigen in the peripheral nerve or adjuvant depots. An analogous phenomenon was described in EAE in which the skin test to myelin basic protein decreased at the height of the disease.¹²

A positive test of specific transformation in the presence of P₂ was clearly demonstrated only with lymph node cells from the P₂ and not the myelin immunised rats. Since the myelin immunised rats had more severe EAN than those immunised with P₂ it is evident that whatever immunological reaction is responsible for the disease may occur in the absence of a positive result with this sensitive *in vitro* test of cell-mediated immunity. This observation is very relevant to the search for evidence of cell-mediated immunity in human acute inflammatory polyneuropathy. It was interesting that P₂ caused some transformation of lymph node cells from adjuvant immunised or normal rats: this might be a non-specific biochemical or mitogenic effect

but might also indicate a universal weak immunological response to a species-specific component or even the autoantigen itself. It would be interesting to discover whether P₂ has a similar effect on other cells, particularly Schwann cells, which proliferate following myelin breakdown with associated release of P₂.

The failure to transfer disease with acute or convalescent serum by intravenous injection and the successful transfer with lymph node cells favour a cell-mediated immune mechanism. Successful passive transfers of EAN with lymph node cells have previously been performed with three rabbits following irradiation of the recipients.¹³ Mild histological lesions of EAN were also reported in guinea-pigs to which had been transferred cells from guinea-pigs immunised with a basic protein derived from human sciatic nerve myelin,¹⁴ an observation which we find difficult to equate with our own demonstration that in the guinea-pig human P₂ basic protein produces predominantly EAE rather than EAN.¹⁵ Injection of cells from rats with EAN directly into the sciatic nerves of rats produced rather more inflammation and demyelination than injection of control cells.¹⁶ EAN serum directly injected into rat sciatic nerve also causes extensive demyelination preceded by Schwann cell damage and accompanied by vesicle formation of the inner myelin lamellae.^{9, 17} These direct injections bypass the blood-nerve barrier and the pathological changes may not represent what occurs in EAN actively induced by the usual emulsion of nerve and adjuvant. In the present experiments EAN was passively transferred with intravenous injections and the absence of antibody to P₂ from the serum of recipients which developed EAN makes it unlikely that our passively transferred disease is caused by circulating antibody. The possibility that B cells and locally produced antibody play a role as well as T cells cannot be excluded without dissection of the cellular mechanisms with B and T cell depleted rats. In EAE similar experiments favour a T cell-mediated mechanism.¹⁸ The failure of 21 day cells to produce transfer could be due to disappearance of "neuritogenic" cells from the lymph nodes: preliminary experiments mixing 9 and 21 day cells have provided no evidence of suppression.

There are reports of antibody to crude nerve extracts¹⁹ and positive *in vitro* tests of cell-mediated immunity to more or less purified nerve extracts in human acute inflammatory polyneuropathy.²⁰ If this human disease is as closely related to EAN as the histological similarity sug-

gests, it will be important to confirm whether cell-mediated immunity to P_2 is present.²¹ Despite the arguments which have been advanced against an important pathogenetic role for antibodies to P_2 in EAN, their presence does provide a readily detected and persistent marker of the autoimmune reaction. If EAN is a relevant model for human inflammatory polyneuropathy it would be expected that antibodies to P_2 would be demonstrable in the serum.

This work was supported by the Medical Research Council.

References

- Kadlubowski M, Hughes RAC. Identification of the neuritogen responsible for EAN. *Nature* 1979; **277**:140-1.
- Smith ME, Forno LS, Hofmann WW. Experimental allergic neuritis in the Lewis rat. *J Neuropathol Exp Neurol* 1979; **38**:377-91.
- Hoffman PM, Powers JM, Weise MJ, Brostoff SW, EAN I. Rat strain differences in the response to bovine myelin antigens. *Brain Res* 1980; **195**:355-62.
- Kadlubowski M, Hughes RAC, Gregson NA. Experimental allergic neuritis in the Lewis rat: characterisation of the activity of peripheral myelin and its major basic protein, P_2 . *Brain Res* 1980; **184**:439-54.
- Suzuki M, Kitamura K, Uyemura K, Ogawa Y, Ishihara Y, Matsuyama H. Neuritogenic activity of peripheral nerve myelin proteins in Lewis rats. *Neuroscience Letters* 1980; **19**:353-8.
- Sarvas HO, Milek DJ, Weise MJ, Carnow TB, Fudenberg HH, Brostoff SW. RIA for the P_2 protein of bovine peripheral nerve myelin. *J Immunol* 1980; **124**:557-64.
- Hughes RAC, Gray IA, Clifford-Jones R, Stern MA. Lymphocyte transformation in the presence of myelin basic protein in multiple sclerosis and control subjects. *Acta Neurol Scandinav* 1979; **60**:65-76.
- Hughes RAC, Stedronska J. The susceptibility of rat strains to experimental allergic encephalomyelitis. *Immunology* 1973; **24**:879-84.
- Saida T, Saida K, Dorfman SH, Silberberg DH, Sumner AJ, Manning MC, Lisak RP, Brown MJ. EAN induced by sensitisation with galactocerebroside. *Science* 1979; **204**:1103-6.
- Saida T, Saida K, Silberberg DH, Brown MJ. Transfer of demyelination by intraneural injection of EAN serum. *Nature* 1978; **272**:649-1.
- Lampert PW. Mechanism of demyelination in EAN. EM studies. *Lab Invest* 1969; **20**:127-38.
- Shaw MC, Alvord EC, Kaku J, Kies MW. Correlation of EAE with delayed-type skin hypersensitivity to specific homologous encephalitogen. *Ann NY Acad Sci* 1965; **122**:318-31.
- Aström KE, Waksman BH. The passive transfer of experimental allergic encephalomyelitis and neuritis with living lymphoid cells. *J Path Bact* 1962; **83**:89-102.
- Abramsky O, Teitelbaum D, Arnon R. Experimental allergic neuritis induced by a basic neuritogenic protein (P_1L) of human peripheral nerve origin. *Eur J Immunol* 1977; **7**:213-7.
- Kadlubowski M, Hughes RAC. The neuritogenicity and encephalitogenicity of P_2 in the rat, guinea-pig and rabbit. *J Neurol Sci* 1980; **48**:171-8.
- Arnason BGW, Chelmicka-Szorc E. Passive transfer of EAN in Lewis rats by direct injection of sensitised lymphocytes into the sciatic nerve. *Acta Neuropathol (Berl)* 1972; **22**:1-6.
- Hahn AF, Gilbert JJ, Feasby TE. Passive transfer of demyelination by EAN serum. *Acta Neuropathol (Berl)* 1980; **49**:169-76.
- Ortiz-Ortiz L, Weigle WO. Cellular events in the induction of EAE in rats. *J Exp Med* 1976; **144**:604-16.
- Nyland H, Aarli JA. Guillain-Barré syndrome: demonstration of antibodies to peripheral nerve tissue. *Acta Neurol Scandinav* 1978; **58**:35-43.
- Hughes RAC. Acute inflammatory polyneuropathy. In: Rose FC, ed. *Clinical Neuroimmunology*. Oxford: Blackwell, 1979; 170-84.
- Sheremata WA, Colby S, Karkhanis Y, Eylar E. Cellular hypersensitivity to basic myelin (P_2) protein in the Guillain-Barré syndrome. *Can J Neurol Sci* 1975; **2**:87-90.