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ORIGINAL ARTICLE

Passive transfer of allergic encephalomyelitis in rats: a tool for drug mechanism studies and detecting late-acting immunosuppressants

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Abstract

1. A strategy is described for evaluating drugs against different phases in the development of an auto allergic disease, experimental allergic encephalomyelitis. It is based on a cell transfer technique whereby the disease is passively transferred with lymphoid cells from actively immunized donor rats to normal syngeneic rats = passive recipients. Drugs may be applied in vivo to either the cell donors or the cell recipients or to cells in vitro whilst in transit; their efficiency being determined by the severity of the passive disease (weight loss, paralysis) in the recipients.
2. Examples are given illustrating the application of these techniques to:
   (a) evaluating the lymphocyte-deactivating activity of various nitrogen mustards in vitro;
   (b) recognizing drugs, e.g. gold derivatives, clofazimine, etc. that are not conventional immunosuppressant (or cytostatic) agents which, when given to the recipient animals, may prevent the expression of the adopted disease;
   (c) comparing some known immunosuppressants for potency, duration of action, etc.;
   (d) demonstrating the versatility of cycloleucine, ICI-47,776, etc.
3. Some merits of the strategy are discussed vis a vis using the local graft-versus-host reaction in rats to search for new drugs.

Keywords Experimental allergic encephalomyelitis (EAE) · Multiple sclerosis (MS) · Pathophoric lymphocytes (PL) · Drug evaluation against PL

Introduction

Experimental allergic encephalomyelitis (EAE) has been intensively studied both as a model of an autoimmune disease and as an analogue of human demyelinating disease, e.g. multiple sclerosis (Paterson 1966, 1968).

It is readily initiated in many species by sensitizing the animals to a preparation of crude myelin (spinal cord, brain) or to purified encephalitogenic proteins or polypeptides, using Freund's adjuvant to evoke a cell-mediated immune response.

For the experimental pharmacologist, this immunopathological reaction can be a valuable tool for evaluating the quality of a novel drug or drug regimen; particularly since at the present time very few drugs, other than established immunosuppressant agents, are known to prevent the onset of paralysis (Rosenthale et al. 1969; Rosenthale 1974). It is, therefore, a

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relatively specific assay, in contrast to many models of inflammation which may respond to counter-irritants, vasodilators, anti-inflammatory agents as well as certain immunosuppressors. To be effective, most of the currently available immunosuppressants must be given during the early stages of EAE development, sometimes even before the encephalitogen is administered. As yet only a few drugs are known which, such as cyclophosphamide, can 'rescue' animals with established paralysis (Paterson and Drobishm 1969) or again such as cyclophosphamide, be administered for the first time as late as 9 days after the encephalitogen (Newbould, unpublished observations).

In searching for new drugs to treat established immunological diseases (Samter 1971) it would be helpful to have a reliable pharmacological assay that would disclose a potentially therapeutic, or late prophylactic, drug such as cyclophosphamide. For a number of reasons, discussed later in this paper, it is rather unlikely that many such drugs will be discovered merely by delaying their time of applications and then seeking to prove their efficacy in moribund animals with such an overwhelming disease as established EAE.

However, by dissecting the development of the disease into two phases—a primary or sensitization phase (without symptoms) and a subsequent clinical phase—it is possible to study drug action on both the initiation of the immunopathology and its expression. This is made possible because the disease may be transferred passively to tolerant or syngeneic recipient animals with lymph node cells (Paterson 1960; Levine and Wenk 1965; Smith and Waksman 1969) or thoracic duct lymphocytes harvested from donor animals in the pre-clinical phase of the disease (Whitehouse et al. 1969). Consequently, drug studies may then be conducted at three levels, namely:

(i) in vivo on animals donating 'pathophoric' (disease-engendering) lymphoid cells;
(ii) in vitro on these cells, whilst in transit to the recipient animals;
(iii) in vivo on the recipient animals, in whom the disease will be expressed unless the drug has been effective at one (or more) of these three stages.

This strategy of drug evaluation closely corresponds to that discussed previously, utilizing a local graft-versus-host reaction (GvHR) in the rat (Beck et al. 1973). There are, however, some distinct advantages in using the EAE, rather than GvHR, for probing drug action:

1. the GvHR lacks a priming phase (against which a drug might be evaluated);
2. the progression of the GvHR cannot be continuously monitored in a given recipient animals (which must be sacrificed for evaluation of the 'disease'), whereas the EAE can be so monitored;
3. the closer relevance of EAE to (auto-)immune pathological diseases in man.

Earlier reports have indicated the utility of passively transferred EAE for drug studies (Newbould et al. 1969; Paterson and Hanson 1969; Gerber et al. 1972). These are amplified in the present communication.

Experimental

Materials

Inbred male rats weighing 200–250 g were used for cell transfer studies. Lewis rats were supplied by Microbiological Associates, Walkersville, Md., Horton laboratories, Oakland, Calif., and the Charles River Breeding Labs. Inc., Wilmington, Mass.: they were sufficiently syngeneic to allow the EAE to be transferred passively between animals obtained from one supplier and those obtained from another supplier in all six possible combinations. Inbred Fischer rats were obtained from Hilltop Lab Animals, Chatsworth, Calif.

In certain experiments, recipient rats were adrenalectomised and maintained on 1% NaCl in the drinking water. Drugs were obtained from the sources specified previously (Beck et al. 1973).

Initiation of EAE

Guinea pig spinal cord (GPSC) was used as the encephalitogen throughout these studies. Freshly isolated GPSC was blended with 4 volumes 0.25% aqueous phenol and frozen at − 20° in 1 ml aliquots. For each experiment a fresh aliquot of frozen GPSC was used. Heat-killed *M.tuberculosis* (mixed human strains C, DT, PN) was obtained from the Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, Surrey, UK, then defatted by three successive extractions with 50% ethanol-ether and stored dry at room temperature. Adjuvants were prepared by very thoroughly grinding 10 mg defatted mycobacteria per ml mineral oil or other oily vehicle and then diluting this (arthritigenic) adjuvant with 9 volumes of the same oil to yield the EAE-adjuvant. Hexadecane, pristane and squalane were sometimes used as alternatives to mineral oil in preparing the adjuvant, being equipotent in inducing EAE (Whitehouse et al. 1974).

Equal volumes of the GPSC and EAE-adjuvant were mechanically mixed in a syringe and thoroughly emulsified by repeatedly forcing the mixture through a double headed 30 gauge needle inserted between two syringes. An emulsion was considered suitable for injection if a droplet

placed on water did not immediately disperse thereon. No exogenous emulsifiers were used to prepare either the adjuvant or the final GPSC emulsion.

Rats were shaved over the lower abdomen, a superficial midline incision made under ether anaesthesia and the inguinal nodes exposed by careful dissection with a pair of fine forceps. Approximately 5–10 µl of the emulsion was injected via a 30 gauge needle into one of the main inguinal nodes on each side of the rat (Newbould 1965). Sufficient material was injected until the milky emulsion could be seen in one of the efferent lymphatics. The incision was then closed with wound clips and liberally douched with 1% solution of picric acid.

Transfer of EAE

At various times within the interval 3–10 days after initiating the disease, the following lymph nodes were collected from 20 or more encephalitogen-sensitized animals terminated by cervical dislocation: cervical, internal axillary, lower abdominal (lumbar), renal and mesenteric nodes. If not too granulomatous, the (injected) inguinal nodes were also excised but kept separately from the other nodes. The nodes were placed directly in chilled sterile Hank's medium and kept on ice. Cells were teased from the nodes by rubbing a pair of thick curved forceps gently, but firmly, over a folded piece of 100 gauge stainless steel cloth, containing a mass of nodes within the fold and immersed in chilled Hank's medium fortified with either 10% (v/v) normal rat serum or 1 mg/ml rat albumin. The cellular dispersion was transferred to centrifuge tubes and again chilled on ice.

After a preliminary brief rapid centrifugation to remove tissue debris, the supernatant was centrifuged for 10 min at 600×*g*. The cell pellet was redispersed in either fresh Hank's medium admixed with 20% (V/v) normal serum or alternatively in Hank's medium fortified with 5 mg rat albumin/ml, for direct infusion into a recipient animal. For in vitro drug studies the cell pellet was suspended in Hank's medium, a cell count obtained and aliquots were added to tubes containing the drug in 1 ml of 0.1 M sodium phosphate, pH 7.4 and sufficient Hank's medium to give a final volume of 5 ml. After incubation at 37° for 15 min, the cells were recovered by centrifugation, resuspended in the Hank's-serum or Hank's-albumin medium (see above) and viability estimated by the trypan blue exclusion test. After a brief, rapid, centrifugation to remove any sludged material, the supernatant was infused into recipient animals and a sample counted for cell content. Cells were infused either via a tail vein over a period of 10 min or via a femoral vein cannula (previously inserted in restrained animals) over a period of 25 min. The latter procedure was preferred to avoid any problems of the animals suffering shock on rapidly receiving a large bolus of chilled cells. A cell infusion prepared from 3 to 5 donor animals (3–15 × 10^8 cells) was needed to ensure a well-developed passive disease in syngeneic 250 g rats.

A few experiments were conducted in which pathophoric cells were obtained from the thoracic duct by described procedures (Whitehouse et al. 1969).

Evaluation of the EAE

Animas were weighed immediately after the cell infusion and at daily intervals thereafter and carefully examined for paralytic symptoms: namely flaccid tail, awkward use of hind limbs, complete paralysis of hind limbs (animals lay half sideways) and very occasionally, death. Other clinical indications were urinary incontinence and faecal impaction. Animals succumbing to disease in the untreated control group usually registered consistent weight loss from the third or fourth day after cell transfer. Disease in the passively sensitized (recipient) animals was nearly always milder (certainly less lethal) than the disease developing in the actively sensitized group of animals. Eventually, nearly all (> 85%) the recipients with active disease recovered full use of their hind limbs, their tails gained tension and they gained weight at an almost normal rate. Both severity and duration of the passive disease were scored: the severity by maximum weight loss (corrected if necessary by weight changes in normal weight-matched animals receiving the same drugs); the duration by the time period during which the tail paralysis was evident.

A third criterion of passive disease-established 'memory' (latent responsiveness)—was also occasionally utilized in drug evaluation. This made use of the finding that animals which had recovered from a relatively severe passive disease, when re-challenged with the original encephalitogen adjuvant emulsion, responded to this active immunization with earlier development of the clinical signs of EAE. The signs of paralysis were usually seen before day 9 while control animals responding to the same batch of encephalitogen adjuvant developed paralysis on or after day 9. The interval for time of onset of paralysis observed in the two groups was sometimes as great as 5 days. In seven such experiments, using a total of 36 animals, the mean time separation for onset of paralysis was 2.7 days. Reduction of this interval to zero indicated that animals, pre-treated with pathophoric lymphocytes from EAE donors, had failed to acquire the capacity ('memory') for giving an early response when challenged (for the first time) with the original encephalitogen. In these experiments, the effect of a drug was not to expunge the memory, but rather to prevent its establishment.

Drug administration

Unless noted otherwise, all drugs were administered daily orally as dispersions.

Results

Factors affecting passive transfer with lymph node cells

In this section and hereafter, 'day N' refers to the number of days after the day of actively initiating EAE by inoculating the encephalitogen in donor animals (day 0). The 'Nth day' refers to the number of days from the time of passively transferring the disease to syngeneic recipients.

Timing of cell transfer

Preliminary experiments indicated that EAE could be transferred passively to adrenalectomized female Lewis rats with 2×10^8 viable node cells harvested from donors inoculated with encephalitogen only 4 days previously, the signs of passive disease (paralysis) being manifest 9 days later. Delaying the time of transfer till days 6–8 provided a higher cell yield from each donor animal and induced signs of paralysis in the recipients by the 7th day (post-transfer) which persisted for at least 4 days. The threshold number of viable cells for a successful transfer to adrenalectomized female recipients weighing 170–200 g appeared to be approximately $1.2 - 1.5 \times 10^8$ cells on day 13.

Animals donating cells eight or more days post-inoculation with encephalitogen usually displayed early signs of EAE, i.e. weight loss and tail paralysis: the accompanying lymphoid involution made it difficult to harvest node cells from donor animals with incipient or established disease. As a compromise, therefore, node cells were routinely harvested during the period days 6–9, on the latest day before most of the donor animals had pronounced disease.

A few experiments were conducted with donors inoculated with encephalitogen and an oily vehicle (squalane) only, i.e. no *Mycobacteria*: these animals usually exhibited severe signs of EAE from day 12 onwards (Whitehouse et al. 1974). On days 5–9, these animals had very small nodes compared with those harvested from animals inoculated with encephalitogen plus a mycobacterial adjuvant. Nevertheless, the disease was transferred as early as day 7 but the optimum time appeared to be in the interval days 11–13.

Nature of recipients

Several comparisons of the sensitivity of normal female and adrenalectomized female Lewis rats indicated that the latter group lost more weight in the 6 days post-transfer and usually showed signs of paralysis 1 day earlier than their normal intact littermates. For these experiments, animals were adrenalectomized three or 4 days before they received node cells. When 5×10^8 node cells from male donors (day 7) were given to weight-matched female and male recipients, no consistent difference in susceptibility was observed. However, when this experiment was repeated several times using 250 g males and 200 g females, the females always manifested a more severe passive disease. Treatment of the recipients immediately after receiving cells with pertussis vaccine (Lilly 200×10^6 *B. pertussis* cells/animal) injected into each hind paw, did not enhance their responsiveness. Inoculating 50 µl of a (non-arthritogenic) dispersion of *Mycobact tuberculosis* in chlorobenzene (10 mg/ml) sometimes provoked a dramatically rapid EAE response in the recipients (paralysis within 3 days) but this effect was not consistent. For routine drug studies, male cell donors receiving encephalitogen plus mycobacterial adjuvant and normal male recipients were used throughout, since the change in weight gain (in control male animals receiving drugs but no lymph node cells) gave a useful clue to potential drug toxicity. The use of adjuvants or adrenalectomy for priming the cell recipients was avoided since they (a) superimposed an unknown physiological burden and (b) generally seemed to enhance the toxicity of certain cytostatic drugs.

Nature of transferred cells

Even massive doses of washed spleen lymphocytes (10^{10}/recipient) failed to transfer EAE, as manifest by weight loss (< 20 g) or signs of paralysis. Differential cell counts indicated that lymph node cells and thoracic duct cells which passively transferred EAE were 95% small lymphocytes. Neither cell-free extracts of node cells (10^{10}) or serum from afflicted animals (10 ml) passively transferred the disease. Incubating node cells in homologous serum (i.e. from sick animals) did not seem to impair their capacity to transfer disease.

EAE was not transferred with 8×10^8 node cells between Lewis and Fischer strains (in either direction or from either of these strains to outbred Wistar rats (CFN, HLW). It was transferred in a much muted form from Fischer (parental) donors to Fischerx Wistar (FxW) F_1 hybrids: however, both the F_1 hybrids and the second parental strain Wistar happened to be rather resistant to the EAE, developing a late (after day 12) and mild disease after being inoculated with encephalitogen plus adjuvant. This combination of Fischer and (FxW) F_1 hybrid had proved highly acceptable for establishing a graft-versus-host response (Beck et al. 1973) indicating the viability of the Fischer graft cells in the hybrids.

Effect of drugs on cell donor animals

For these studies, the donor animals were treated continuously from day 0 till the day of transfer. In our limited experience, only known cytostatic or lymphopenic drugs

Table 1 Effect of drugs applied after cell transfer upon the EAE development in the recipient animals

Drug (Code No.)	Dose Mg/Kg	DSI[a]		DSI control		Toxicity Index[b]	Signs of paralysis
		6th day	9th day	6th day	9th day		
Cyclosamide	7.5	0.3	N.D.	3.5	N.D.	3	0
	10	0	1.5	3.0	7.5	3	0
Isophosphamide	30	1.5	3.0	3.0	7.5	2	0
Chlorambucil	4	1.0	3.0	3.0	7.5	0	0
Cycloleucine (W-4267)	12.5	4.0	5.0	5.0	8.0	0	+
	25	1.0	1.5	5.0	8.0	5	0
	37.5	1.0	2.0	5.0	8.0	12	0
1-Aminocyclohexan-1-carboxylic acid (W-4708)	60	4.0	8.3	5.0	8.0	0	++
Procarbazine.HC1 (Ro-4-6467)	37.5	5.0	6.5	5.5	8.5	2	+
	50	2.0	2.0	4.0	7.0	11	0
ICI-47,776	10.0	1.2	3.0	3.0	6.5	0	±
	12.5	0	0	1.5	4.5	6	0
EN-3638	50	0.5	0	2.0	4.0	0	0
	100	0	0	2.0	4.5	6	0
Na, Aurothiomalate[c]	8.0	1.5	2.0	3.0	6.0	4	±
Azauridinetriacetate	200	2.0	3.5	1.5	3.5	3	+
Clofazimine	50	1.0	1.5	2.5	4.5	N.D.	±
(Pre-treat)[d]	50	0	1.0	2.5	4.5	10	0
Alanosine	25	1.0	2.0	3.0	5.0	5	0

Drugs administered once daily to recipients from the day of transferring cells from donor animals (harvested 7-9 days after inoculating encephalitogen). Development of disease in concomitant untreated animals is recorded as positive control. Weight changes due to drug alone yielded a toxicity index (see below). Development of EAE was scored by a disease severity index (DSI) of an animal on any day, computed as a sum of points assigned as follows: 1 per 10 gm weight loss (corrected for effect of drug alone); 1 for tail paralysis; 1 for rear end paralysis; in addition, 1 point is allotted for each 4 days duration of tail paralysis, and 1 for mortality. A score of 5.0 would normally be the maximum on the 6^{th} day, and a score of 8 or 9 (depending on mortality) on the 9^{th} day

N.D. not determined

[a]Mean values on $6^{th}/9^{th}$ days after cell transfer with 2 or more animals/group

[b]Toxicity index = % weight loss of animals (no cells) with drug alone, computed as loss of body weight from day of transfer (= 1st drug dose) to 6th (or 9th) day/body/weight on day of transfer × 100. Normal 22–260 gm animals receiving neither drug nor cells showed average weight gain of 5–7% and 8–10%, respectively, over 67 and 9 days

[c]After initial loading dose of 25 mg/kg, all doses given i.m

[d]See text; control animals received arachis oil in all experiments with clofazimine

affected development of EAE at this stage. This was readily discerned by the size of the nodes at day 7 and the low cell yield obtained therefrom (60% yield compared with that from untreated donors) e.g. after daily dosing with 5 mg/kg cyclophosphamide, 0.5 mg/kg dexamethasone, 1.0 mg/kg betamethasone, 1.75 mg/kg 6-thioguanine, 10 mg/kg ICI-47,776 (Franklin et al. 1966). These and related compounds also inhibited the lymphoproliferative response i) after inoculating Lewis rats with an arthritigenic adjuvant (Newbould, unpublished observations) and (ii) after establishing a graft-versus-host reaction in FischerxWistar F_1 hybrid rats (Beck et al. 1973).

A massive collection of nodes from cyclophosphamide-treated donors provided sufficient cells for administration to six adrenalectomized female rats (i.e. the most sensitive recipients in our experience). None of these recipients showed signs of EAE on receiving 1.2×10^8 cells/180 gm recipient.

Donor animals treated with sodium aurothiomalate (25 mg/kg/day i.m.) or clofazimine (50 mg/kg/day orally in arachis oil) had somewhat enlarged nodes (or spleens) at the time of transfer (day 7) but provided cells that were almost equipotent with those harvested from untreated donors for passively transferring the EAE. Signs of EAE developed 1 day later than was to be expected, in all animals receiving cells from clofazimine-treated donors.

Effect of drugs on the recipient animals

For these experiments, groups of three animals were dosed with drugs to establish any effect of the drug alone on weight gain. Other animals received both cells (from day 7 to 9

Table 2 Drug action on pathophoric node cells in vitro, determined by their subsequent ability to passively transfer EAE

Drug/procedure	Conc.mM	DSI[a]		DSI (control)		Paralysis in recipients
		6th	9th	6th	9th	
Radiation—200R—500R	– –	0.5 0.5	D.	1.5	5.0	0
Bis chlorethylamine HC1	0.01	0.5	N.D.	6.0	N.D.	0
Mechlorethamine. HC1 (HN2)	0.05	0	0.5	4.5	6.0	0
Chlorambucil	0.1	0	0	2.0	5.0	0
Melphalan		01		0.1		1.5
Mannomustine	0.05 0.1	0 0	3.0 1.0	2.8	6.5	±
Cyclophosphamide (CPA)	2.5	4.0	7.0	4.0	7.5	++
CPA + liver homogenate	[b]	0	0	4.0	7.5	0
CPA + Ascorbate/Fe++/EDTA	[c]	0	0	0	3.0	0
Pyridofuroxan	0.15	0	N.D.	3.0	N.D.	0
Maleimide	0.08	1.5	2	4.0	8	0
Succinimide	0.3	4.0	7	4.0	8	++
Dithiodinicotinic acid	1.0	5	9	6	9	++
4-Vinyl pyridine	1.0	0	0	1.5	4	0

Data are mainly mean of duplicates (2 controls, 2 experimental animals per observation)

N.D. not determined

[a]See Table 1

[b]Yielding alkylating metabolites equivalent to 30 μM HN2, as determined with nitrobenzylpyridine (see Whitehouse et al., 1973)

[c]Yielding alkylating metabolites equivalent to 90 μM HN2

donors) and the drugs—usually at least two animals received cells and any one level of drug. Weight loss, indicative of EAE onset, was computed by differences in the weight changes of these two groups from the time of transfer to the 6th and 9th day thereafter.

Data recorded in Table 1 are from experiments, the findings of which were confirmed in at least one independent cell transfer experiment. Of the 12 drugs listed, only two proved to be completely ineffective: they were the homologue of cycloleucine with a cyclohexane ring (W-4708) and azauridine triacetate (Azaribine®), a potent anti-psoriatic agent. None of the 10 active compounds, except possibly EN-3638* was really effective at truly subtoxic doses. The toxicity of procarbazine (Natulan®) at effective doses was especially noteworthy.[1]

Effect of treating cells in transit with drugs or radiation in vitro

The strategy of these experiments was to:

(i) treat populations of lymphocytes (usually node cells) known to transfer EAE with various drug or physical regimens in vitro and

(ii) then transfer them to virgin syngeneic recipients to see if they retained their pathophoric activity, i.e. ability to initiate signs of EAE in their adopted host. In essence, we used an in vivo bioassay to probe the effects of drug action in vitro that were not readily apparent otherwise.

Only treated cell populations that were no less viable (as evidenced by exclusion of trypan blue) than untreated aliquots of the same population were infused into the recipients. Potential toxicity to cells (not immediately altering their viability) was detected by reduction in the incorporation of uridine-5-^{3}H or mixed amino acids-^{14}c into RNA and cell protein, respectively. (Whitehouse and Doskotch 1969) on incubating the drugs with aliquots of the pathophoric node population. All drugs employed in vitro were routinely checked for their potential toxicity to lymphocytes in vitro by incubating them with rabbit or rat thymocytes and thymidine-6-^{3}H to determine the ID_{50} for inhibiting tritium incorporation into DNA (Whitehouse and Doskotch 1969).

Whenever practicable, further drug studies were conducted at ID_{20} (or lower) concentrations.

[1] EN 3638 is 6-hydroxyphthalaldehydic acid, 0-(p.chlorobenzyl) oxime. Clofazimine (B-7663, Lamprene®) was somewhat more effective when given for seven days prior to cell transfer, rather than after cell transfer; paralleling the effect of this drug in suppressing adjuvant arthritis (Currey ans Fowler 1972).

Table 2 enumerates some drugs studied by these procedures. Low levels of radiation (500R) abolished the pathophoric activity of both node cells and thoracic duct cells, contrasting with the much high levels (1000R or more) needed to deactivate splenic lymphocytes and prevent them from initiating a graft-versus-host reaction in tolerant recipient rats (Beck et al. 1973).

Subtoxic levels of several mustards effectively deactivated pathophoric node cells (Table 2). Cyclophosphamide was completely inert in this respect. However, when cyclophosphamide was (a) decomposed by boiling in dilute acid for 10 min and then neutralized, or (b) oxidized non-enzymically with ascorbate-ferrous ions –EDTA (Udenfriend et al. 1954) or (c) metabolized in vitro by fortified rat liver preparations (Whitehouse et al. 1973), the products obtained in each instance were effective deactivators of node lymphocytes. In these latter experiments, the controls included incubating cells with the Undenfriend system or liver homogenates without added cyclophosphamide.

Some problems were encountered in that ascorbate alone partly deactivated phathophoric node cells. Similar attempts to monitor pharmacologically active cyclophosphamide metabolites in bile (Whitehouse et al. 1973) were completely frustrated by the fact that bile from control animals (not receiving cyclophosphamide) contained lymphocyte-deactivating activity. These findings were all the more remarkable since the same levels of ascorbate or biliary products (devoid of cyclophosphamide metabolites) had no effect on some other rat lymphocyte populations, including node cells, when subsequently bio-assayed by the graft-versus-host reaction (Beck et al. 1973). Many drugs were found to have no lasting effect on the cells, even though they are known to profoundly affect biopolymer synthesis in lymphocytes or had proved effective in preventing the development of EAE. These included 0.1 mM mitomycin C, 0.2 mM puromycin, 0.5 mM flazalone, R-760 (Whitehouse 1971) and all the compounds listed in Table 1 (Tested at 0.1 mM or 0.5 mM) except chlorambucil, which was effective in vitro and in vivo. Drugs binding irreversibly with cellular thiols such as maleimide, 4-vinyl pyridine (Friedman et al. 1970) or pyridofuroxan (Ghosh and Whitehouse 1969) had a lasting effect on the cells. Reversible thiol inhibitors such as 6,6′- dithiodinicotinic acid (Mehrishi and Grassetti 1969) on the other hand did not. All the effective compounds, with the exception of vinyl pyridine, deactivated splenic lymphocytes as monitored in the graft-versus-host assay (Beck et al. 1973).

Mechlorethamine and maleimide were also found to inhibit pathophoric cells collected from (i) thoracic duct lymph of normal EAE donor animals on days 8–10, and (ii) the lymph nodes of donors inoculated with encephalitogen and squalane, i.e. without *Mycobacteria* on days 10–11.

Drugs action on 'memory'

A feature of treating the cell-recipient animals with cyclophosphamide (10 mg/kg) and cycloleucine (37.5 mg/kg) was that the 'memory trace' of the disease was never established, as determined by a subsequent challenge with encephalitogen. This was the case even when the animals showed minimal signs of EAE, particularly weight loss (over and above that observed in controls receiving drugs but no cells). By contrast, procarbazine (50 mg/kg) and azauridine triacetate (200 mg/kg) did not apparently prevent the recipients being sensitized by the passive transfer of node cells, so that after being challenged with encephalitogen they gave an early active EAE response (the doses of procarbazine had been sufficient to prevent overt paralysis in the recipients).

Discussion

Practical considerations

A key feature of these experiments is that semi-quantitative data are obtained with relative ease: there is no need to resort to tissue sectioning and histological methods to evaluate the disease status of treated animals and their untreated controls. This modest advance is possible using a highly inbred and encephalitogen-susceptible rat strain (such as Lewis or Fischer) and is certainly assisted by initiating a vigorous disease in the donor animals through the lymph node injection technique (Newbould 1965). These methods certainly do not lend themselves to screen large numbers of compounds. There is the continual problem of assigning significance to experiments involving only a few animals for each drug at each dose (in vivo or in vitro).

However, if the experiments are used to probe when—or where—the drug may primarily act in preventing or ameliorating the establishment of an autoallergic injury in a qualitative sense, then adequate answers may certainly be gleaned from this approach. There may be little advantage in studying the effect of drugs directly on 'committed' lymphoid cells in vitro before transfer to the recipients, as described here, when the almost identical stratagem can be employed with far less trouble using the graft-versus-host reaction (GvHR) as the in vivo bioassay for in vitro drug action (Beck et al. 1973; Whitehouse et al. 1973). However, as noted in the Results section, there are a few agents (including X irradiation) which may affect cells transferring EAE more readily than they affect cells initiating the GvHR. This is all the more surprising since much larger cell numbers must be treated with drugs to affect EAE in an individual recipient than to affect the GvHR (ca. 5×10^8 vs. 5×10^7, respectively): a differential response to the same amount of drug may in fact mean that cells transferring EAE

are indeed much more drug-sensitive than those initiating the GvHR. On the other hand, other drugs (notably HN2) were required at higher concentrations in vitro to deactivate cells transferring EAE than to prevent a subsequent GvHR (even with the same node cell population taken from Fischer rats). Since higher cell numbers were required in the EAE experiments this might indicate that a given amount of drug had to be incorporated per cell, regardless of which pathogenic activity was ultimately expressed.

This problem of balancing the amount of drug with the number of cells was also encountered occasionally in in vivo studies. Thus, in one series of experiments, 25 mg/kg/day cycloleucine prevented EAE development after infusing 0.9×10^8 cells whereas 50 mg/kg was required to prevent the disease on infusing 6.7×10^8 cells/recipient.

General pharmacological considerations

Cycloleucine and ICI-47, 776 would seem to be rather versatile immunosuppressant like cyclophosphamide, in affecting both early and late stages of EAE development. Other features of their immunosuppressant activity in rats are discussed by Rosenthale et al. (1972) and Davies (1968).

The brief period of drug treatment employed in these in vitro experiments, followed by the long incubation periods in vivo, effectively precluded the recognition of reversible drug action. Only irreversible agents (mustards, thio-blocking agents) were readily detected by this procedure of treating cells in vitro. This approach has proved useful nevertheless for monitoring the relative efficacy of a variety of mustards, particularly putative metabolites of cyclophosphamide (Whitehouse et al. 1972) for their lymphocyte-deactivating activity. Some experiments in which the in vivo viability of mustard-treated cells was carefully monitored (Yu and Whitehouse 1973) suggest that the mustards were not just poisoning the cells and causing them to die shortly after transfusion but were indeed selectively toxic (Albert 1973).

The extravagance and labour of these experiments are certainly justified when the results permit a positive reassessment of marginal drugs.

For reasons alluded to in "Introduction" and spelled out below, only exceptional drugs may be detected when their application in vivo is delayed for several days after inoculating the encephalitogen, i.e. well into the post-sensitization phase or even waiting until the disease is actually expressed. In these present experiments at least three classes of drugs were detected unequivocally by this passive transfer technique, namely anti-arthritic gold preparations, the anti-leprosy drug clofazimine (Currey and Fowler 1972) and a novel salicylate derivative, EN-3638 (see footnote, p14 this MS). All these compounds had rather marginal activity at completely non-toxic doses when evaluated by their ability to prevent homologous EAE development, i.e. initiation, amplification and expression within the one animal (Gerber et al. 1972; Fowler et al. unpublished observations). By focussing the assay on the events between the generation of a pathophoric population of committed lymphocytes and the subsequent target tissue injury, it is thus possible to find anti-injury agents which may not be anti-aetiological (in the sense of being able to successfully prevent the immunological priming events that initiate the ultimate pathology).

The novelty of these three classes of drugs is indicated by their relatively weak anti-proliferative activity e.g. in preventing node hypertrophy after immunization or the graft-versus-host reaction in related rat strains (Beck et al. 1973; Fowler, unpublished findings).

Pathological considerations

It has been our experience that even when a powerful immunosuppressant agent such as cyclophosphamide (or sustained depletion of circulating lymphocytes via thoracic duct drainage) is applied to prevent disease initiation, it may in fact do no more than delay the onset of symptoms—probably because another population of pathophoric cells may be generated in vivo to replace that deleted (by previous drug treatment). Consequently as long as the encephalitogenic determinant remains within the lymphoid tissues, a pathogenic response is probably bound to develop unless the animals are kept in a state of almost complete, certainly chronic, immunosuppression. Another factor mitigating against facile control of disease development in encephalitogen-inoculated animals beyond the priming phase is the tremendous amount of target tissue (all parts of the nervous system containing myelin) accessible to the population of committed leukocytes surviving an immunosuppressant regimen.

The analogy of the Sorcerer's Apprentice and his broom is appropriate. The pressure of the flood of committed cells, continually engendered while the original encephalitogen persists, is effectively removed by taking just so many of these cells from one or several donor animals and then studying their drug sensitivity either in vitro or in vivo after adoptive transfer to recipient animals which contain ample target tissue but none of the original encephalitogen (i.e. guinea pig spinal cord). In this context, lymphocyte deactivation is amenable to study with minimal complications from continuing lymphocyte activation. Far less massive (and, therefore, less toxic) amounts of drug may be needed to delete, or inactivate, the sensitized population of cells in the passive recipient than in the active donor.

To some extent this same 'ideal' is attainable when the active population is a viable graft which needs no prior immunological stimulation, as exploited in graft-versus-host reactions (see Beck et al. 1973). However, as pointed out above, there are still advantages to studying EAE by passive

transfer—notably the recognition of drugs such as EN-3638, clofazimine and gold preparations which frankly 'perform poorly' as anti-lymphoproliferative agents and, therefore, do not rate highly in the graft-versus-host assay. They presumable control the 'adopted EAE' by interfering with one or more of the molecular and cellular events which underlie the expression of an immunopathy.

To designate late immunosuppressants, as in the title of this article, may be rather a misnomer if for example, they predominantly act on aggressive monocytes/macrophages or extracellular inflammogenic processes.

Regardless of nomenclature, they might serve as 'leads' from which to develop new drugs to treat established immunoinflammatory diseases. Their discovery may validate the methodologies reported here.

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Appendix

Further comments by the third author (M Whitehouse)

Nearly half a century later, this report is finally in print after its inception by Brian Newbould in 1969.

In 1974 it was considered too biological for a pharmacological journal and too drug-oriented for an immunological journal. The editors of both these leading journals were not interested in the new concept implied in this quest for more logical drug design; particularly targeting potential pathophoric lymphocytes and controlling their pathogenic activities through a range of mechanisms e.g. anti-sensitisation or suppressing populations of sensitised leukocytes or their subsequent roles in inducing tissue injury. The advent of this journal *Immunopharmacology* 27 years ago has certainly made it easier to bridge the interface between immunopathology and pharmaceutics. We must be grateful to all those who made it happen and to the three publishers who successively sustained the journal after its launch in 1991.

An updated overview of EAE for drug evaluation

A reviewer kindly suggested some updates for using experimental auto allergic encephalomyelitis (EAE) as an animal model for multiple sclerosis (MS) (Constantinescu et al. 2011) and noted some limitations of this model (See Baxter 2007; Denic et al 2011; Lassman 2018). These are primarily based on the discrepancy between knowing the nature of the auto-antigen(s) used to trigger EAE in rodents (and other experimental animals) and the supposedly unknown entities that both trigger and sustain the clinical spectrum of MS.

Ebringer and his colleagues have recently indicated that an infection might trigger MS, particularly as a source of bacterial antigens, eg some derived from *Acinetobacter*, that may cross-react immunologically with human myelin (Ebringer 2015). This has prompted a search for classic and novel antibiotics targeted to control *Acinetobacter baylyi* infection including some traditional medicines sourced from Africa, Australia and India (Sirdaata et al 2015; Mandeville and Cock 2018; Cock and Cheesman 2018). But knowing the identity of an initiating agent may not be sufficient for treating established disease.

Several of the current therapies for MS (Broadley et al 2015) were developed after being shown to suppress EAE in animals. However some other treatments that effectively suppressed EAE development in animals have provided little or no clinical benefit for patients with MS. Yet others now used to treat MS have not been so effective in controlling EAE in animals at subtoxic doses eg dimethyl fumarate (DMF) effective in mice (Schultz-Topphoff et al 2016) but not in rats.

In summary, the EAE model certainly has limitations for validating new clinical agents to treat MS. But as used in this original study from UCLA, it does allow dissection of disease development and drug evaluation at each of the three stages (I–III).

$$\text{Antigen} \xrightarrow{\text{I}} \text{Sensitisation} \xrightarrow{\text{II}} \text{Auto-intolerance} \xrightarrow{\text{III}} \text{Neurological injury}$$

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