Antilymphocyte Serum and Tissue Culture Used to Investigate Role of Cell-mediated Response in Viral Encephalitis in Mice

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British Medical Journal, 1970, 4, 210-212

Summary: Antilymphocyte serum given to suppress selectively the cell response to Langat virus in Swiss albino mice prolonged the average survival times. In vitro, lymph-node cells from virus-immunized mice were strongly cytotoxic for syngenic non-neuronal brain cells infected with virus. The implications of these findings are discussed, with particular reference to the concept of autoimmunity.

Introduction

The demonstration by Weigand and Hotchin (1961) that lymphocytic choriomeningitis virus, which invades the central nervous system of mice, causes no disturbance in the absence of an immune response was a dramatic example of how the host's immune responses may sometimes contribute to the pathogenesis of infectious disease. The relevance of some such mechanism to the pathogenesis of experimental infections of mice with the T.P. 21 strain of Langat virus was reported by Webb *et al.* (1968). Mice irradiated with 500 rads before intracerebral injection of virus had more prolonged viraemias and higher brain virus titres than controls and yet survived longer and died with little clinical or histological evidence of encephalitis. The death of the control animals from paralysis appeared to be related to the earlier production of specific antibody and cellular infiltrates, rather than to the concentration of virus in the brain. These experiments, however, did not indicate the relative importance of the cellular and humoral responses, since irradiation depresses both.

Whereas antibody levels can be measured without difficulty it is notoriously difficult to assess the cell response in mice. With some antigens, however, antilymphocyte serum will suppress both responses if given before the antigen but will not affect antibody production if given 24 hours after the antigen (Martin and Miller, 1968). In this study we have used these effects of antilymphocyte serum to assess the relative importance of the cellular and humoral mechanisms in the pathogenesis of Langat encephalitis, and have correlated our findings with observations on the effect of Langat-sensitive lymph-node cells on virus-infected non-neuronal cells of the central nervous system in vitro.

Materials and Methods

Langat virus, both mouse-passaged (T.P. 21) and plaque-adapted (T.P. 77), were used in these experiments, and the results obtained were the same with both viruses. The viruses were obtained from Dr. C. E. Gordon Smith, of the Microbiological Research Establishment (M.R.E.), Porton. Trypsin dispersed whole brain cell suspensions from Swiss A₂G mice were prepared on coverslips in Sykes Moore chambers (Illavia, 1970) and infected at the time of seeding with approximately 4.5 log doses of T.P. 21 virus; these were used five days after infection when 4.5 log doses of virus were being produced by the cells.

Antilymphocytic serum (A.L.S.) was obtained from Burroughs Wellcome & Co. (B.W.) and from the M.R.E. The A.L.S.-treated mice used in experiment 1 were injected subcutaneously within 12 hours of birth with 0.05 ml. of A.L.S. They were subsequently given 0.05 ml. on day 4, 0.1 ml. on

days 8 and 11, 0.15 ml. on day 15, 0.2 ml. on day 10, and then 0.25 ml. twice weekly until death. They were inoculated with T.P. 21 virus at the age of 5 weeks. In experiment 2, 0.25 ml. of A.L.S. was given subcutaneously 24 hours and five days after intracerebral inoculation of virus in 5-week-old mice.

Lymph nodes were used as a source of lymphocytes and were taken from mice which had recovered from intraperitoneal injection of either T.P. 21 or T.P. 77 and injected on subsequent occasions with the relevant virus emulsified with an equal volume of Freund's complete adjuvant (Difco). The lymphocytes were added at a concentration of 150×10^6 per ml. in such a way that they gravitated on to the infected monolayer, except in one group in which the chambers were inverted before the cells were added.

Results

Experiment 1.—The effect of A.L.S. (B.W.) administered subcutaneously twice weekly since birth on the course of T.P. 21 encephalitis in 5-week-old mice was investigated. Forty A.L.S.-treated mice were inoculated intracerebrally with 2.7 log doses mouse intracerebral lethal dose₅₀ (ICLD₅₀) of T.P. 21 virus at the age of 5 weeks. The day of death of 29 of these was recorded, and the remainder were killed on day 9 or 10, for histological examination and estimation of brain virus content and the serum haemagglutinin inhibiting antibody levels. A similar group of 40 control mice were given the same virus dose (at 5 weeks), but no A.L.S. The average survival time of the control mice was 7.4 days (after inoculation), whereas that of the A.L.S.-treated mice was 8.6 days. This was shown to be statistically significant ($P < 0.001$). The incidence of paralysis in the A.L.S.-treated group was significantly less, and delayed in time as compared with the controls: all mice of both groups had brain virus titres between 6.0 and 6.8 log doses, this value being unaffected by A.L.S. treatment. No haemagglutinin inhibiting antibody to the virus was detected in the serum of the treated mice, however, and the cellular infiltrate and perivascular cuffing which is very prominent in the control mice was minimal, though there was more neuronal damage attributable to a direct effect of the virus.

Experiment 2.—The effect of A.L.S. administered 24 hours and five days after infection on the course of T.P. 21 encephalitis in 5-week-old mice was investigated. One hundred mice were given 2.3 log doses of T.P. 21 virus intracerebrally. Of these, 37 were treated with the A.L.S. (M.R.E.) and 10 with the A.L.S. (B.W.) used in experiment 1. A further 37 mice were not given A.L.S. The survival time of all mice in these three groups was recorded. The remaining mice were killed on day 8 or 9 for histological study, estimation of brain virus content, and serum haemagglutinin inhibiting antibody levels. The average survival time of the control mice (after inoculation) was 8.3 days whereas that of the A.L.S.-treated mice was 10.1 days. The figure was identical with both batches of A.L.S. Statistical analysis showed $P < 0.001$. The titres of virus in the brains of the treated mice did not differ significantly from those of the controls (average, 6.7 log doses on day 8). All mice tested showed evidence of haemagglutinin inhibiting antibody, and the titres of treated and control mice were identical (ranging from 1/20 to 1/80). The histological changes were identical to those seen in experiment 1.

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This experiment showed that A.L.S. administered after the virus did not stop the production of antibody, and yet the survival time was prolonged. Thus prolonged survival appeared to correlate not with the suppression of antibody but with the absence of the cell-mediated response.

Experiment 3.—The effect of lymph-node cells from mice specifically immunized with virus on virus-infected syngenic non-neuronal brain cell monolayers in vitro was investigated. Six experiments were performed with similar results (see Table). The effect was the same whether the lymphocyte

Comparative Effect of Virus-sensitized Lymph-node Cells, Normal Lymph-node Cells, and Similar Cells from Animals Inoculated with Freund's Adjuvant Only on Normal Mouse Brain Tissue Culture and Virus Infected Tissue Cultures

	% Sheet Intact at 72 Hours	
	Infected	Control
Controls (no lymph-node cells)	100 100	100 100
"Sensitized" lymph-node cells	<50 <50 <50 <50	75 90 60
Normal lymph-node cells	99 90	99 100 99
Sensitized lymph-node cells. Chambers inverted. No contact	100 100	100 100
Lymph-node cells from mice injected with adjuvant only	100 100	100 100

donors were immunized with T.P. 21 or T.P. 77 virus. Controls were included using lymph-node cells from mice which had received Freund's complete adjuvant without virus. These lymphocytes had no cytotoxic effect. The findings were that lymph-node cells from mice which have recovered from infection with Langat virus and which have received booster doses of virus in Freund's complete adjuvant have a pronounced cytotoxic effect on virus-infected monolayers and a detectable effect on non-infected cells. These effects were seen only when there was contact between the lymph-node cells and target cells.

Discussion

Experiments 1 and 2 suggest that immunosuppression with A.L.S. prolongs the lives of mice infected intracerebrally with Langat virus by reducing the cell-mediated response rather than by reducing specific antibody formation.

The cytotoxic effect of virus-sensitized lymph-node cells on virus-infected cell monolayers shown in experiment 3 may provide a model for the apparent damaging effect of the cell response in the in-vivo system. The mechanism may be similar to that of target-cell destruction, which has been reported previously (Rosenau and Moon, 1961). The arboviruses, of which Langat is one, are among those which "bud" from cells when they are being released. In this process modified cell membrane is incorporated into the coat of the new virion. During the process viral antigens are present on the cell surface and there may be new antigens expressed on the cell wall even before the budding process begins. This certainly occurs during the transformation of cells by some tumour viruses and both Zlotnik (1968) and Illavia and Webb (1970) have shown the proliferative effect of some virus strains, including Langat virus, on non-neuronal brain cells both in vivo and in vitro.

It has been suggested in the past that the target-cell destruction mechanisms may play a part in the elimination of cells undergoing malignant change or of virus-infected cells (Burnet, 1968). We feel that the essential point may be that these mechanisms evolved in order to destroy autologous cells which become antigenic to the host, whether as a result of mutation, or infection inside the cell, or modification of the

cell surface from outside. Virus infection, however, must be much the most frequent cause of such a change in antigenicity.

If, therefore, suppression of target-cell destruction contributed to the prolonged survival of the A.L.S.-treated mice one must explain an apparent paradox. It is widely accepted that whereas antibodies may have an important defensive role during the viraemic phase, it is cellular immunity which combats virus infections that have become established in the tissues. A cellular immunity deficiency syndrome has been described which includes a normal incidence of bacterial infection and normal levels of antibody to both viruses and bacteria, but abnormal susceptibility to virus infections (Di George, 1965). The protective role of the cell response has also been reviewed in several experimental systems (Allison, 1967). Destruction of an infected cell is the surest way to interrupt the replication cycle of the virus. Flick and Pincus (1963) concluded that delayed hypersensitivity caused the primary lesions of vaccinia in rabbits and so blocked dissemination of the virus.

Thus the cell response appears to protect against viruses in most cases, and yet in our experiments its suppression prolonged survival. We suggest that when a virus is localized in a few expendable cells destruction of these cells results in termination of the infection without excessive damage to the host. When, however, circumstances lead to a relatively late production of sensitized lymphocytes, many non-expendable cells may be rendered antigenic before the cell response strikes. Then the organ in question—in our experiments, the brain—may suffer irreversible damage.

It is worth considering that such a mechanism could easily masquerade as an "autoimmune" disease if one were unaware of the presence of a virus. There may be many latent micro-organisms in apparently normal tissue which are sterile by conventional techniques of isolation (Rogers *et al.*, 1967). An excellent illustration is the case of the N.Z.B. mice, which for several years were said to suffer from a hereditary autoimmune disorder (Russell *et al.*, 1966). It now seems that it may be the antigen which is abnormal and not the response (Holborow and Denman, 1968). It may be significant that virus particles have been demonstrated (East *et al.*, 1967).

In experiment 3, however, lymph-node cells from mice which had recovered from the infection and received booster doses of virus in Freund's complete adjuvant had a weak toxic effect on the cells even in the absence of virus. There was no infectious virus in the lymph-node suspensions from immunized mice, but it is still possible that there was enough viral antigen present to cause release of "lymphotoxin" in the chambers, as reported by Williams and Granger (1968) and Ruddle and Waksman (1968). The absence of a cytotoxic effect when the chambers were inverted so that the lymphocytes did not contact the monolayer does not entirely eliminate this possibility, since lymphotoxin diffuses very poorly. Lymphocytes from animals immunized with Freund's complete adjuvant alone had no effect. A more likely explanation is that there was sensitization to some other antigen from the brain.

The existence of such antigens in nervous tissue, capable of giving rise to "autosensitization," is widely accepted (Waksman, 1959), and experimental "autoallergic" encephalitis has been produced in Swiss albino mice (Olitsky and Yager, 1949). As stressed above, however, until the origin of the antigen has been clearly defined one cannot be sure that it is a true autoantigen. Whatever its origin, we suggest that in our experiments it has either been released from the brain as a result of cell damage or more probably been incorporated in the outer coat of the budding virus. Possibly antigens from the tissues are particularly immunogenic when presented to the host's immune mechanisms in a modified form in the coat of a virus. Cells infected with Langat virus continue to release virus for long periods (Illavia and Webb, 1970), and hence there may be prolonged release of potent antigen into

the circulation. This release must also occur during many trivial infections of man, and so may explain why sera and lymphocytes from a small percentage of normal individuals have a cytotoxic effect on glial cells *in vitro* (Berg and Källén, 1964). One can suggest a similar explanation for the much more pronounced cytotoxic effects of lymphocytes from patients with multiple sclerosis.

We are grateful to Dr. S. J. Illavia and Miss G. E. Fairbairn for their invaluable help with the tissue cultures used in these experiments, and to Mr. E. Bowen, of the M.R.E., Porton, for the sample of antilymphocyte serum. The reports on the histological sections of the brains were kindly given to us by Dr. D. G. D. Wight, of the department of pathology, St. George's Hospital, London. The work was made possible by a generous grant from the Wellcome Trust.

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Agreement Between Clinical and Radiological Diagnosis

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British Medical Journal, 1970, 4, 212-214

Summary: Agreement between radiological and clinical diagnosis and the incidence of unexpected findings has been used as an indicator of the diagnostic contribution of radiology. The overall incidence of agreement was 33% — 533 out of 1,604 x-ray examinations. The wide variation in agreement in different radiological examinations is clearly related to the pathological nature of disease and the limitations of radiological techniques. If diagnostic radiology is to be effective its application rather than interpretation in the clinical situation must be emphasized in training programmes.

Introduction

The contribution of radiology to clinical diagnosis is not in doubt, but few attempts have been made to quantitate it outside the field of gastrointestinal disease (Cooley, 1961; Rawson, 1965; Cook, 1966). Agreement between the radiological and initial clinical diagnosis and the incidence of unexpected findings are used in this paper as indicators of the radiological diagnostic contribution. This method has obvious limitations as there is no final pathological confirmation of the diagnosis, but it has the merit of simplicity and is valid as a good approximation.

Method

All the requests for initial x-ray examinations of the chest, abdomen, skeletal structures, skull, and for intravenous pyelography, cholecystography and cholangiography, barium enema and barium meal examinations were assessed during a six-week period. The following information was tabulated: (1) the department of origin of the request (medical, surgical, or other specialty—including ophthalmology, E.N.T., dermatology, obstetrics and gynaecology, paediatrics—and general

practitioners who have direct access to the x-ray department); (2) whether the radiological interpretation agreed with the clinical diagnosis as stated on the request card; (3) whether additional pathological lesions not anticipated by the clinician were observed—these were categorized as being (a) of interest or (b) of significance in terms of patient management; and (4) in cases where the clinician had clearly indicated that the examination was of a routine nature or that he anticipated a normal report and this was confirmed radiologically the request was designated "normal."

Of the 1,846 requests included in the study, 997 were from the medical department, 617 from the surgical department, and 232 from the other specialties, including 111 from general practitioners. Casualty requests were not included in the study.

In this survey the observer is heavily dependent on the accuracy of the clinical diagnosis on the original request card. Fortunately, a parallel study of follow-up requests (unpublished data) indicated that the original diagnosis was usually retained for subsequent examinations, supporting the view that it was likely to be correct in most cases.

Results

"Normal" Requests

Of 242 requests received, 223 were for chest x-ray examination (125 medical, 71 surgical, and 27 from other specialties). Thirteen of the remainder were "medical" and were distributed evenly among the other examinations. The proportion of "normal" to total chest x-ray examinations for each referring group was greatest in the surgical group and least in the medical group, an incidence of 52 and 22%, respectively. The other specialties had an incidence of 38%. Four of the patients who had "normal" requests were found to have a significant additional lesion (one bronchial carcinoma, one metastases from renal carcinoma, one idiopathic cardiomegaly, and one pulmonary cyst).

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