DISCUSSION

It can be concluded from these experiments that the acquisition of a temperature-sensitive defect causes a decrease in virulence. A similar result has been found with *ts* mutants of Semliki Forest virus in mice, and rabbit-pox virus in rabbits (Fenner, 1968). Nevertheless, the mutants must replicate in the mouse lung to the extent that they can induce high titres of antibody directed against the viral haemagglutinin.

It was not possible to correlate virulence with the physiological characters of the mutants (Mackenzie and Dimmock, in preparation), nor with their genetic locations (Mackenzie, 1969). Of the four attenuated mutants, only *ts* 7 and *ts* 18 have had defective functions assigned to them; *ts* 7 has been found to be defective in haemagglutinin, and *ts* 18 is believed to be defective in neuraminidase. Both *ts* 17 and *ts* 19 synthesized haemagglutinin and neuraminidase at the restrictive temperature. Other mutants which have defects in their haemagglutinin (*ts* 1, *ts* 6, and *ts* 11) or in neuraminidase (*ts* 5) were totally avirulent. There was also no correlation between cut-off temperature and virulence, in contrast to *ts* mutants of Semliki Forest virus (F. J. Fenner, personal communication, 1968).

It has been shown by Schulman, Khakpour, and Kilbourne (1968) that antibody directed against neuraminidase has a protective effect in the mouse lung, and this provides a possible explanation for the protective effect of *ts* 1 and *ts* 6 to subsequent challenge by WSNp despite low antihaemagglutinin antibody levels.

From the results in this communication and genetic results to be published elsewhere (Mackenzie, 1969) a hypothetical, but rational, procedure can be put forward for the production of an attenuated live influenza vaccine along the lines previously suggested by Fenner (1968). A basic vaccine strain could be produced from a recent influenza isolate (*A*₂ strain) in one of two ways: either by obtaining a strain with multiple *ts* mutations by recombining suitable single *ts* mutants with defects in non-structural genes (double and multiple *ts* recombinants could be verified by back-crossing) or by heavy mutagenic treatment of an existing *ts* stock. The former technique has

the advantage of being able to select for adequate growth capacity and stability of each specific *ts* lesion to be included in the basic vaccine strain. Such a strain could be maintained easily by standard genetic methods.

The actual vaccine strain could be obtained by simple recombination between the basic vaccine strain and each new antigenic variant by selecting for the new antigenic variant with the *ts* characteristics. Once again the stability could be verified by back-crossing to ensure the inclusion of all *ts* lesions.

The value of this procedure is threefold. A basic vaccine strain having been obtained, the first attribute is the ease with which a suitable recombinant could be isolated (recombination occurs at a high frequency with influenza); secondly, the actual vaccine strain could very quickly be tested for reversion and stability; and, finally, it is envisaged that up to 1,000 vaccine doses could be prepared from the yield of a single egg, in contrast to about a single dose from each egg of the currently used killed vaccines. Of course, the efficacy of such a live vaccine could be determined only by trials in man.

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Antilymphocytic Serum in Experimental Allergic Encephalomyelitis

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Summary: Antilymphocytic and antithymic sera (raised in rabbits) are highly potent in preventing or alleviating experimental allergic encephalomyelitis in guinea-pigs. Good results are obtained when treatment is begun as late as 10 days after challenge. No protracted disease was seen from the use of antilymphocytic serum. Attempts to use this serum therapeutically once the disease was clinically established gave equivocal results. Antilymphocytic serum appears to have a non-specific stressor effect as well as its specific action, and possibly this adrenocorticotrophic-hormone (antiphlogistic) effect may ameliorate a developing encephalitis. The presence of an antimacrophagic factor in antilymphocytic serum may be important in increasing local reaction to injection of the serum and perhaps also in diminishing the appearance of haematogenous elements in the nervous lesions.

INTRODUCTION

Experimental allergic encephalomyelitis is a disease readily produced in guinea-pigs by inoculation of whole brain or an

encephalitogenic factor with Freund's complete adjuvant (Robertson *et al.*, 1962; Caspary and Field, 1965). A great deal of evidence now links the experimental disease with sensitization of lymphocytes, and it is considered to be a form of delayed hypersensitivity (Paterson, 1966). Since it is also regarded by many as a model for multiple sclerosis the influence of antilymphocytic serum and antithymic serum on the condition is of importance. The present work sets out the results of experiments aimed at the prevention and treatment of the disease.

MATERIALS AND METHODS

ANTILYMPHOCYTIC AND ANTITHYMIC SERUM

Cervical and mesenteric lymph nodes (or thymus lobes) were removed from healthy adult Hartley guinea-pigs of both sexes, cleaned, cut up, and teased in chilled Hanks's solution. After washing, the dissociated cells were filtered through a 100 stainless steel mesh and then through an 80 mesh. Final suspensions were made up to contain about 10^8 cells per inoculum (1.5 ml.) in Hanks's solution. A dye exclusion test showed about 80% viability of lymph node cells and 95% of thymic cells.

Large adult albino rabbits were immunized by intravenous injection of the cells. This was repeated 10 days later, and five days after that the animals were bled. Further injections were carried out about every two to three weeks and 25-50 ml.

of blood was collected five or six days afterwards. Sera were shaken twice with guinea-pig red cells to remove haemagglutinins, and lymphocyte agglutination titres were estimated. These rose to as much as 1:1,200 after four inoculations.

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

Experimental allergic encephalomyelitis was produced in Hartley guinea-pigs (400–500 g.) by inoculation of 10 µg. of human encephalitogenic factor (Caspary and Field, 1965) together with Freund's complete adjuvant (H37Ra—Difco) in a single 0.1-ml. dose into the dorsum of the right foot. Animals were weighed and examined daily at the same time, and the first sign of illness was usually a sudden loss of 20–40 g. in weight. All animals were examined histologically at the end of the experiment, sections being taken coronally through the brain and medulla and longitudinally through the spinal cord.

Animals were assigned a score (Disease Index) on the scheme suggested by Kies *et al.* (1957) which takes into account both the neurological disturbance and the histological picture. In this system five is the maximum score and is given to an animal which dies (or has to be destroyed because of severe paralysis) accompanied by full histological changes. Lymphoid tissues were also taken for histological examination, but the results are outside the scope of the present work. Blood leucocyte counts were also made at intervals (ear specimens).

PREVENTION EXPERIMENTS

The following groups of animals were set up:

Controls.—(1) Saline-treated, beginning simultaneously with sensitization (11 animals). (2) Normal rabbit serum treated, beginning simultaneously with sensitization (12 animals). (3) Normal rabbit serum, beginning one week after sensitization (12 animals).

Experimentals.—*Antilymphocytic serum*: (4) 0.5 ml. daily, beginning simultaneously with sensitization (6 animals); (5) 1 ml. daily, beginning simultaneously with sensitization (5 animals); (6) 1 ml. daily, beginning 10 days after sensitization (12 animals). *Anti-thymic serum*: (7) 0.5 ml. daily, beginning simultaneously with sensitization (5 animals); (8) 1 ml. daily, beginning simultaneously with sensitization (6 animals); (9) 1 ml. daily, beginning one week after sensitization (12 animals).

THERAPEUTIC EXPERIMENTS

In these experiments animals were allocated for treatment as controls (with normal rabbit serum) or with antilymphocytic serum alternately at the first appearance of signs. This procedure was rightly adhered to, since it is not possible to say at the onset of signs in any given animal whether it will rapidly deteriorate or undergo only a minor recoverable disturbance. Any conscious selection of animals might well introduce bias. In the first experiment six control animals were matched against 13 antilymphocytic serum ones; in the second experiment 18 controls against 17 antilymphocytic serum.

RESULTS

With the particular encephalitogenic factor in use the picture of experimental allergic encephalomyelitis in the guinea-pig is remarkably consistent. Results are set out in Tables I and II. A distinct difference was seen between animals treated prophylactically with saline and those treated with normal rabbit serum, the latter method having a distinct sparing effect (referable, as appears below, to its mildly stressor effect). The slightly protective effect of normal rabbit serum was also brought out when the length of "incubation period" following sensitization was compared for saline and normal rabbit serum treated animals. For the former it was 12.3 ± 0.3 and for the latter 15.1 ± 0.5 days ($P < 0.001$).

A daily dose of 1 ml. intraperitoneally was able to suppress experimental allergic encephalomyelitis completely when treatment was begun at the same time as sensitization, while 0.5 ml.

had a distinct though lesser effect. Even 7 or 10 days after sensitization treatment had an obvious sparing effect.

TABLE I

	Treatment	No.	Score
<i>Controls</i>			
Saline	Simultaneous	11	$4.6 \pm 0.1^*$
N.R.S.	Simultaneous	12	$3.6 \pm 0.1^{\dagger}$
N.R.S.	1 week late	12	$4.1 \pm 0.4^{\ddagger}$
<i>Experimentals</i>			
A.L.S.	Simultaneous daily, 0.5 ml.	6	$1.3 \pm 0.4^{\S}$
	Simultaneous daily, 1 ml.	5	0
	1 ml. daily 10 days late	12	$2.6 \pm 0.5^{\parallel}$
	Simultaneous daily, 0.5 ml.	5	$3.2 \pm 0.2^{\S}$
A.T.S.	Simultaneous daily, 1 ml.	6	0
	1 ml. daily 1 week late	12	$0.7 \pm 0.2^{\parallel}$

* $P = 0.001$. $\dagger P = 0.001$. $\ddagger P = 0.05 - 0.025$. $\S P = 0.005 - 0.001$. $\parallel P = 0.005 - 0.001$.

TABLE II.—Treatment at Onset of Illness

		Alive	Dead	Total
Experiment 1*	Controls	0	6	6
	A.L.S.	8	5	13
	Total	8	11	19
	Controls	13	5	18
Experiment 2†	A.L.S.	10	7	17
	Total	23	12	35
<i>Figures of Leibowitz et al. (1968b)</i>				
At day 1‡	Controls	4	6	10
	A.T.S.	10	1	11
	Total	14	7	21
At day 10§	Controls	0	10	10
	A.T.S.	4	7	11
	Total	4	17	21

* Fisher's exact test $P < 0.05$. $\dagger P = 0.6 - 0.7$. $\ddagger P < 0.05$. $\S P = 0.2$.

Histologically, those animals which scored 0 had no lesions at all in the nervous system, and in those animals where perivascular cuffing was found it was distinctly reduced in degree.

While antilymphocytic serum produced a pronounced fall in blood lymphocytes within a few hours of inoculation, this became progressively less as injections were continued daily. Control animals (treated with normal rabbit serum) tended to have a lymphocytosis at about 10 days, but this was masked in the animals treated with antilymphocytic serum (Fig. 1). Polymorphonuclear leucocytes showed an early rise following injection of antilymphocytic serum but soon returned to normal levels (Fig. 2).

Experiment 1 to test the therapeutic effect of antilymphocytic serum suggested that it had reduced mortality ($P < 0.05$). In experiment 2, however, no beneficial effect at all was found, though sera from the same animals were used.

FIG. 1.—Effect of normal rabbit serum (—) and antilymphocytic serum (----) on lymphocyte cells in experimental allergic encephalomyelitis.

FIG. 2.—Effect of normal rabbit serum (—) and antilymphocytic serum (----) on polymorphonuclear leucocytes in experimental allergic encephalomyelitis.

DISCUSSION

The use of antilymphocytic serum for suppression of hypersensitivity reactions of "delayed" type has recently been reinvestigated by Waksman *et al.* (1961), who among other models used experimental allergic encephalomyelitis in guinea-pigs. They found marked suppression of the tuberculin and contact allergic reactions, and of delayed skin reaction to purified diphtheria toxoid. So far as experimental allergic encephalomyelitis was concerned, they observed that antilymphocytic serum injected before or during the incubation period resulted in a "pronounced delay in the onset of the disease or possibly, in some cases, in its complete prevention." Experimental allergic encephalomyelitis was not notably milder in those animals in which lymphocytes were markedly depressed than in those with less pronounced depression. They also noted that lymphocytic depression in the blood tended to become less marked as daily injections of antilymphocytic serum continued—that is, there appeared to be an escape phenomenon, similar to that described in the present work.

More recently Leibowitz *et al.* (1968a) studied the effect of antilymphocytic serum (actually antithymic serum) before sensitization, during the "incubation period," and as a therapeutic agent once paralysis had appeared. They found that both early and late treatment before onset of signs reduced the incidence of experimental allergic encephalomyelitis and that the serum could exert a therapeutic effect if given to animals already showing definite paralysis. Thus they found that 2 ml. of antilymphocytic serum intraperitoneally on alternate days reduced mortality within 24 hours from 6 out of 10 to 1 out of 11 ($P < 0.05$). From their data, however, it appears that, while 10 out of 10 control animals were dead by the 10th day, 7 out of 11 animals treated with antilymphocytic serum had also died. The Fisher exact test shows $P = 0.2$, so that this difference has a one-in-five chance of being fortuitous. Under these circumstances more experiments are called for, and at the moment it can only be said that antilymphocytic serum perhaps has some therapeutic effect once nervous disease is established in guinea-pigs.

A few animals in which experimental allergic encephalomyelitis had been prevented clinically by antilymphocytic serum were observed for nearly three months after the serum had been stopped (at 21 days), but no recrudescence of the disease and none of the extensive demyelinating lesions described by Leibowitz *et al.* (1968b) were observed.

In discussing the mode of action of antilymphocytic serum, Waksman *et al.* (1961) remarked on the varying degrees of weight loss and anorexia shown by guinea-pigs treated repeatedly with antilymphocytic serum and noted that injection of

it subcutaneously produced "a brownish pink oedema of the body wall lasting more than 24 hours" with extensive polymorph infiltration extending into subjacent muscle layers. They were disinclined, however, to attribute much significance to a possible adrenal-mediated stressor effect of antilymphocytic serum, since they claimed "correlation of decreased reactions with lymphopenia." The stressor effect of this serum has been investigated more recently by Field and Cook (1969), who found that normal Hartley guinea-pigs given 1 ml. antilymphocytic serum daily intramuscularly showed considerably more weight loss than control animals given normal rabbit serum. Moreover, the blood cortisol level was greatly raised in the former group, but reverted to normal as the injections were continued.

Some early work of Woodruff and Forman (1950) recognized a non-specific component in the lymphocyte fall induced in rats by injection of antilymphocytic serum (raised in another strain of rat). Repetition of these experiments in adrenalectomized rats (Woodruff, 1960) showed that the leucocyte count remained unchanged after the injection of either immune or control serum. Further work (Woodruff *et al.*, 1966), however, suggested that a non-specific adrenal-motivated reaction was not the major mechanism operative in response to antilymphocytic serum injection. Nevertheless, it does seem possible that the rise in blood cortisol level within four hours after injection of antilymphocytic serum (Field and Cook, 1969) might be responsible for the protective effect reported by Leibowitz *et al.* (1968a, b). The ability of antilymphocytic serum to inhibit peripheral manifestations of delayed hypersensitivity (tuberculin reaction: contact dermatitis to oxazolone and dinitrochlorobenzene) has been described by Waksman *et al.* (1961) and Turk and Willoughby (1967). "This peripheral effect may be due to interference with the action of sensitized lymphocytes or to some non-specific effect, such as that which has been shown to depress the acute inflammation reaction to turpentine in the guinea-pig skin" (Leibowitz *et al.*, 1968a).

The antilymphocytic serum used in the present experiments contains an antimacrophagic factor which immobilizes these cells but does not destroy them (Field and Hughes, 1969). It is possible that the severity of reaction at the injection site might in part result from immobilization of macrophages, and that diminished perivascular infiltration in the brain might in some measure be due to impeded migration of haematogenous elements which have been shown to be important in developing brain lesions (Konigsmark and Sidman, 1963).

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