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Langat virus encephalitis in mice

II. The effect of irradiation

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In view of the known suppressive action of irradiation on the immune response (Taliaferro, 1957) the following series of experiments have been carried out in an attempt to further evaluate the role of antigen-antibody reactions in the development of viral encephalomyelitis.

MATERIALS AND METHODS

These were as described in the previous paper (Webb, Wight, Platt & Smith, 1968). The same strain of mice was used but they were 22–25 days old in the majority of the experiments. Mice of ages ranging up to 8 weeks were used to obtain a base line for irradiation dosage. Since in these preliminary experiments a standard age seemed to be more important than a standard weight, mice 22–25 days old were used as their age could be more exactly estimated.

Antibody studies. IgM (19S) and IgG (7S) levels were measured by treating the mouse plasmas with 2-mercapto-ethanol (2-ME) after acetone extraction as follows. One volume was incubated for 2 hr. at room temperature with an equal amount of buffered saline containing a final concentration of 0·1 M 2-ME. Another volume was similarly incubated with an equal volume of normal saline. The 2-ME-treated extracts were then dialysed overnight in the cold against buffered saline containing 0·02 M iodoacetamide. Otherwise the method was as in the previous paper.

Irradiation was by gamma rays from ^{60}Cobalt sources. The experiments were conducted between June 1965 and May 1967. During this period the cobalt sources were renewed and in order to obtain uniformity all calculations are based on the activity of the various sources on the 1st January 1966, a decay factor based on a half life of 5·25 years being employed. The doses were checked under experimental conditions by a Baldwin–Farmer substandard dose meter with a Perspex build-up cap added to give electron equilibrium, bolus being added to simulate the body of a mouse.

Up to seven mice were irradiated at the same time. Each mouse was held in a plastic tube 3·1 cm. overall diameter, with a wall thickness of 0·2 cm., closed at either end by perforated rubber bungs. The mice were held in a jig on the central axis of a twin-headed cobalt unit so that they were in a single vertical plane with

a tube of bolus above and below the top and bottom mouse respectively. The dose variation for the top and bottom mouse as compared with the dose received by the middle mouse was 95·5%. Irradiation was given from both sources simultaneously, the beams being 24 × 24 cm. The dose rate was approximately 500 rads in 4·5 min.

Data from all our irradiation experiments have been plotted graphically and the irradiation LD 50 for 21-day-old mice, under our experimental conditions, was approximately 580 rads.

RESULTS

Table 1 shows how irradiation increased the sensitivity of the mice to peripheral infection with virus. Following intracerebral (i.c.) inoculation there was a higher virus titre in the irradiated animals compared with the controls. With intraperitoneal (i.p.) inoculation while all irradiated animals given dilutions from 10^{-3} to 10^{-8} died, only 15 of 36 controls over a similar dose range died (varying from 0 to 4 in each group of 6). As the i.p. inoculum given at each dilution was approximately 7 times as large as that given i.c., irradiation raised the i.p. virus titre

Table 1. *Titrations in control and irradiated mice* 3 *weeks old inoculated i.c. or i.p. with Langat virus.* 500 *rads were given* 24 *hr. before inoculation*

	Virus given i.c. 0·03 ml.				Virus given i.p. 0·2 ml.	
	Irradiation		Controls		Irradiation	Controls
Dilution of virus	Deaths	ADD*	Deaths	ADD	Deaths	Deaths
10^{-3}	—	—	—	—	6/6	4/6
10^{-4}	—	—	—	—	6/6	2/6
10^{-5}	—	—	—	—	6/6	2/6
10^{-6}	—	—	—	—	6/6	3/6
10^{-7}	5/5	10·0	5/5	9·6	6/6	4/6
10^{-8}	4/5	12·5	4/5	9·5	6/6	0/6
10^{-9}	3/5	12·3	0/5	—	—	—
10^{-10}	1/5	16·0	0/5	—	—	—
LD 50	$10^{-9·2}$	—	$10^{-8·4}$	—	$\geqslant 10^{-8·5}$	—

* Average day of death of mice.

approximately to the i.c. titre and gave it a clear end-point as compared with a very scattered one in the controls. This experiment also showed that, although the virus titre was higher in the irradiation group inoculated i.c., the average day of death (ADD) of the mice at the dilutions 10^{-7} and 10^{-8} was later than in the control group although the total mortality was identical. The control animals in these experiments died an unquestionable paralytic death, whereas the majority of the irradiated mice merely became hunched up with ruffled fur but no clinical evidence of paralysis was observed.

Table 2 compares the brain virus titres when approximately 100 ICLD 50 of virus was given i.c. either with or without preceding irradiation. Again, there was a significant prolongation of the ADD of the irradiated mice ($P < 0·01$). To make the experiment more complete a further five mice had been added to each group and treated in a similar manner except that they were killed on the seventh day

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after infection. Their brains were divided sagittally: one half was titrated for virus and the other half preserved for histological examination. Table 2 shows that although the titres of the brains from the irradiated group were, if anything, higher than those of the controls, the brains of the irradiated mice showed no histological changes, while the control brains all showed the characteristic changes of encephalitis.

Table 2. *The average day of death, brain virus titres and brain histology on the 7th day after inoculation in mice given* 500 *rads irradiation and then inoculated* 24 *hr. later with* 100 *ICLD* 50 *of Langat virus i.c., compared with mice given the virus only*

	Nos. in group	Deaths (%)	ADD in days	Brains, day 7: Mean virus titre (log_{10})	Brains, day 7: Histological encephalitis
Group 1. Irradiation and virus	14+5	100	10·6	6·8*	0/5
Group 2. Virus only	14+5	100	8·9	5·6†	5/5
Group 3. Irradiation only	7	0	—	—	0/7

* Range 6·3–7·2. † Range 3·7–6·5.

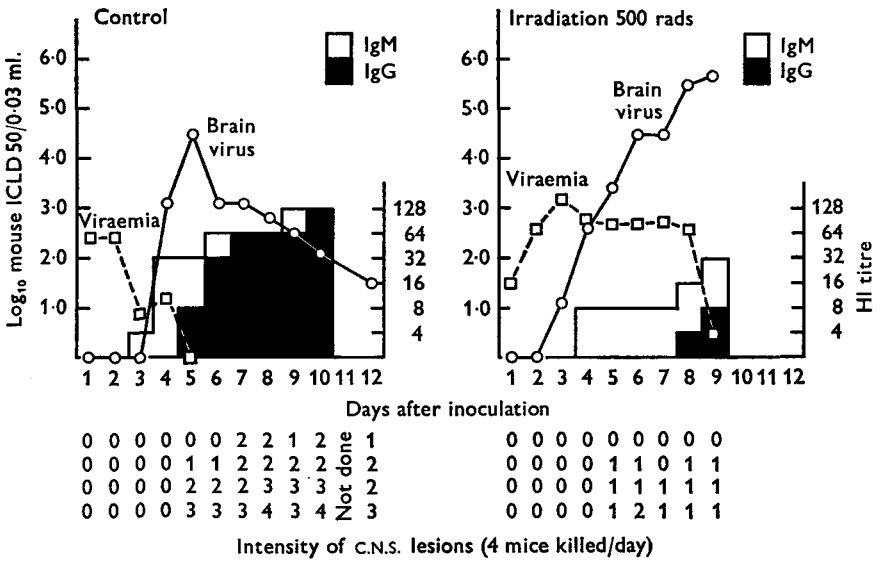

Fig. 1. Viraemia, brain virus, IgM and IgG titres and intensity of histological lesions following intraperitoneal inoculation of 1,000,000 ICLD 50 of Langat virus, 24 hr. after whole body irradiation with 500 rads.

Figure 1 depicts the results of an experiment in which approximately 1,000,000 ICLD 50 of virus was inoculated i.p. into control animals and into a group which had been irradiated with 500 rads 24 hr. previously. Following infection four mice were killed from each group on each day up to the tenth and on the twelfth day; all the irradiated group had died by the tenth day. The bloods and sagittal

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half brains of each of these groups of four mice were then pooled and titrated for virus. The remaining half brains were examined histologically, and the bloods tested for antibody. During the first 3 days the brain virus titre was either negligible or well below the blood virus titre. From the fourth day the brain virus titres exceeded the viraemia titre, but while in the control animals the brain virus reached its maximum on the fifth day and then subsided, in the irradiated mice the brain virus titre continued to rise and reached levels higher than even the maximum of the control mice. Figure 1 also shows the reciprocal serum antibody titres; there was little, if any, delay in the appearance of IgM antibody in irradiated mice although its titre may have been lower and it may have persisted longer. There was, however, a delay of about 3 days in the appearance of IgG antibody in irradiated mice and their overall antibody response was clearly diminished. In both cases, the viraemia began to fall with the appearance of detectable IgM antibody and ceased shortly after IgG became detectable.

A similar experiment in irradiated mice was performed using a much smaller virus dose (100 ICLD 50). The results were similar except that viraemia was not detected until the third day. The titre then remained at about 1000 ICLD 50 per 0·03 ml. until the ninth day, the one before death. There was a gradual rise in the brain virus titre from the fifth day until death as in the previous experiment.

Figure 1 also incorporates the histological findings. The inflammatory lesions of encephalitis were first seen in the control mice on the fifth day. The striking feature of all the brains of mice which had been previously irradiated was the almost total absence of histological encephalitis compared with the controls, though the brain virus titre was higher in the irradiated mice. The majority of these brains differed in no way from those of normal uninfected mice. However, neuronal changes, an infrequent finding in the control mice, were seen as often in the irradiated group. They were seen most commonly in the granular layers of the cerebral cortex and in the hippocampus and consisted of shrinkage and eosinophilia of the cytoplasm with pyknosis or karyorrhexis of the nuclei.

In an experiment (Table 2) where approximately 100 ICLD 50 was inoculated i.c. and the brains were taken on the seventh day, the findings were similar. The brains of the mice which had been irradiated showed no evidence of the expected inflammatory lesions of encephalomyelitis, while the brains of the controls showed typical encephalomyelitis. The brain virus titres tended to be higher in the irradiated mice, although the difference was not significant.

Table 3 shows the result of an i.p. titration in mice 3 weeks old which had received different doses of irradiation 24 hr. before inoculation. A dose of 300 rads or greater increased the sensitivity of the mice to the maximum when all mice in all dosage groups died. The change in behaviour was most marked between 50 and 100 rads.

To bring together these results with those in the previous paper (Webb *et al.* 1968) some experiments were performed in which irradiation was followed by administration of pre- and post-infection ascitic fluid. Table 4 shows the results of one such experiment. The administration of post-infection fluid i.v. on day 3 did not significantly alter the survival of mice which had been given virus i.c. However,

in those which had been given virus i.p. two-thirds of the group survived and the remainder had a prolongation of survival time. Paralysis occurred in this group after the same interval as in mice which had been given virus and post-infection fluid but had not been irradiated. As expected, there was a 100% death rate in the group which had received virus i.p. followed by pre-infection fluid i.v. An extra number of mice in each i.p. virus group were kept for histological examination: nine mice from each were killed on the twelfth day. Of the mice which had

Table 3. *Proportion of 3 weeks old mice dying after i.p. inoculation of dilutions of Langat virus, given 24 hr. after the mice were subjected to different doses of irradiation*

Dilution of virus	Controls	Rads of irradiation 24 hr before virus					
		50	100	200	300	400	500
10^{-2}	6/6	—	—	—	—	—	—
10^{-3}	4/6	3/6	4/6	5/6	6/6	6/6	—
10^{-4}	2/6	0/6	3/6	6/6	6/6	6/6	6/6
10^{-5}	2/6	2/6	3/6	5/6	6/6	6/6	6/6
10^{-6}	3/6	2/6	6/6	5/6	6/6	6/6	6/6
10^{-7}	4/6	4/6	5/6	6/6	6/6	6/6	6/6
10^{-8}	0/4	2/6	4/6	5/6	6/6	6/6	6/6

Table 4. *Comparison of the effects of 0·1 ml. i.v. of undiluted post- and pre-infection human ascitic fluid given on day 3 after giving Langat virus, 500 ICLD 50 i.p. or 50 ICLD 50 i.c., to 4–6 weeks old mice which had been given 500 rads of irradiation before infection*

Post-infection ascitic fluid				Pre-infection ascitic fluid			
Virus given i.c.		Virus given i.p.		Virus given i.c.		Virus given i.p.	
ADD*	Deaths	ADD	Deaths	ADD	Deaths	ADD	Deaths
9·3	23/23 (100)	17·6	3/9 (33)	8·3	23/23 (100)	13·2	9/9 (100)

Figures in parentheses indicate percentages.
* ADD = Average day of death.

received post-infection fluid six had no lesions and three had minimal lesions. Of the mice which had received pre-infection fluid one had no lesions, the remaining eight had lesions of grades 1 or 2. As in the previous experiments the lesions were atypical in that neuronal destruction was more prominent than is usual in association with such mild inflammatory changes. Thus, although there was the usual suppression of inflammation in the pre-infection fluid group, directly related to the irradiation, there was even less inflammation in the group which had received post-infection fluid after whole body irradiation and virus.

DISCUSSION

These experiments have shown that following irradiation antibody formation was delayed and partially suppressed. Irradiation also significantly increased the average survival time in those mice which had been given a small dose of virus i.c.,

and in those groups infected i.p. viraemia was prolonged and the brain virus titre continued to rise until death. However, the mode of death of the irradiated mice whether infected i.c. or i.p. was essentially non-paralytic. Therefore it seems reasonable to associate this lack of clinical neurological disturbance with the alteration in development of antibody. In addition the histological changes of encephalitis were also suppressed. Since the changes seen are largely due to the presence of mononuclear cells the destruction of these cells by irradiation must have some relevance to this.

Several workers have reported that irradiation may lead to increased susceptibility to infection with certain viruses (Imam & Hammon, 1957; Quilligan *et al.* 1963; Schneck & Berkovitch, 1965), and irradiation has been shown to inhibit antibody formation (Taliaferro, 1957) and interferon production (Julien & de Maeyer, 1966). The combined inhibition of antibody and interferon could well account for the prolongation of viraemia in the irradiated mice.

In our experiments, the viraemia in the control mice ended between the fifth and sixth day after infection and clinical neurological involvement began on the seventh day. Virus appeared in the brain on the fourth day, reached a peak on the fifth day and then gradually declined but was still present on the twelfth day. In contrast, viraemia in irradiated mice remained near its maximum from the second till the eighth day, and the brain virus titre rose steadily from the third day until death (Fig. 1). The higher brain titre of the irradiated mice may have been due to interferon suppression, but may have been apparent rather than real: in brains ground up after the appearance of antibody, a proportion of the virus would be neutralized before titration. Figure 1 shows that antibody, probably IgG, whether naturally produced or, as shown in the previous paper (Webb *et al.* 1968), artificially administered, plays a major part in the termination of the viraemia. Kundin (1966) and Tasker, Miesse & Berge (1962) have drawn similar conclusions with other arboviruses.

Langat virus in mice, in common with many other arboviruses, clearly has two distinct multiplication cycles: the first causing a high titre of virus in the blood, the second causing a high titre of virus in the brain. The lymph nodes draining the inoculation site area appear to be the site of primary virus multiplication (Malkova & Frankova, 1959 et seq.) and these and other lymph nodes are the likely sites for early antibody production (Litt, 1964, 1967). At the time of the main peak of viraemia antibody is circulating but as it will be rapidly combined with virus it is very difficult to detect. Evidence from other workers (Notkins, Mahar, Scheele & Goffman, 1966; Ashe & Notkins, 1966) suggests that considerable quantities of circulating virus may be, at this stage, combined with antibody but not neutralized. The virus-antibody complexes at this stage are small but as more antibody appears and combines they become larger and virus is neutralized. When antibody becomes detectable it is excess antibody, virus is then rapidly neutralized as it is released, and viraemia is no longer detectable. However, in irradiated mice antibody is diminished or delayed so that detectable viraemia is markedly prolonged. Depression of interferon production may have contributed both to the prolongation of viraemia and to the rather higher brain virus titres following irradiation.

The inflammatory changes in encephalitis are probably allergic reactions to virus-antibody complexes. These could be expected to occur where large complexes are deposited on the walls of small vessels, at points of serum filtration from the vessels and at places in the central nervous system (CNS) where virus released from cells encounters diffusing antibody. These are thus represented by aggregations of mononuclear cells in the meninges and perivascular spaces, together with proliferation of the glial elements at sites where there is primary neuronal damage from virus multiplication in the cells.

A small dose of specific antibody administered i.p. or i.c. before inoculation of virus was sufficient to protect the animals completely, even against large virus doses (Tables 2 and 7, Webb *et al.* 1968). In these circumstances the virus is presumably neutralized before it can disseminate and probably before it can even reach the regional lymph node. Thus, no detectable viraemia develops, the host does not fall ill, and there is no immune response. Administration of antibody after virus inoculation almost completely suppressed viraemia (Table 1, Webb *et al.* 1968). During the first 24 hr. the virus will have multiplied in the local lymph node and will have been spread via the blood to small numbers of cells throughout the body. Concomitant with this, antibody production will have begun in sensitized lymph nodes. Antibody administered at this stage will greatly slow the spread of virus from cell to cell by combining with a high proportion of released virus. Thus virus production and also the stimulation of antibody will be suppressed. Several types of antibody are formed during the course of a virus infection, some early and some late. The antibody formed later may well be the most important one because this antibody is at least partly antibody to the virus-antibody complexes formed earlier (Najjar, Robinson, Lawton, & Fidalgo, 1967) which are antigenically different from virus alone and which may contain an element of antibody to the host antibody itself. It may well be this antibody which combines together the antibody-coated virus particles and small complexes to produce complete neutralization of the virus and the larger complexes which we believe are responsible for the inflammatory aspect of the disease. Thus, under the experimental conditions, one would expect this late-type antibody and an accumulation of large virus-antibody complexes rather later in mice given antibody than in control mice. Also, because of the early general suppression of host antibody response, CNS virus multiplication can go ahead probably at a higher level for a longer time. The combination of these two factors could well cause the delay and the increase in incidence of paralysis and a greater intensity of lesions in certain areas. Both in animals given antiserum and in controls the meningitis and perivascular cuffing would be accounted for by a type of allergic reaction to virus-antibody complexes from the blood stream; similarly, glial reactions around areas of neuronal damage would be explained by an allergic reaction to complexes formed between diffusing antibody and released virus. The reaction may in fact be on a quantitative basis and the factor that determines which individual animal develops clinical encephalitis may depend on a critical numerical relationship between virus and antibody at a given time. We have shown that all inoculated mice have histological evidence of infection in the central nervous system. Virus was recovered from all but two of

the brains; despite this, only a small proportion showed clinical signs of encephalitis.

We have not satisfactorily explained the relatively sudden non-paralytic death of the irradiated mice around the tenth day. However, their uniform death may have been due entirely to virus multiplication, uninhibited by either antibody or interferon, with consequent destruction of functional cells both in the CNS and other tissues. It is important to note that at the time of death of these animals both IgM and IgG were present and the possibility of some acute severe form of antigen-antibody reaction has not been excluded as a cause of death. If this is relevant, then it would appear that IgG is the more important antibody since the animals die within 48 hr. of the appearance of this.

The reason for the apparent reduction in paralysis rate on days 5 and 6 is not clear. It is known that the handling of mice produces stress which stimulates the release of steroids (Chang, 1965). This could well be advantageous to the mice at this stage by controlling oedema from the result of an antigen-antibody reaction. We have experiments in hand that tend to confirm that steroids given at this stage are beneficial. Also the addition of antiserum itself into the circulation at this time may alter circulating antigen-antibody complexes in a way beneficial to the mouse. This is also under investigation.

The site of inoculation of the antiserum may be relevant. The mice inoculated with antiserum i.p. had an increase both in frequency and intensity of lesions in the hind brain and spinal cord and in particular an increase in frequency and thickness of the perivascular cuffs in these areas. The serum had been inoculated into dermatomes supplied by the lumbar nerve roots. This situation appears to be analogous to that in poliomyelitis where trauma to a limb can lead to that part becoming the site of maximum paralysis.

The experiments in which animals were irradiated and then given antiserum may throw further light on the problem. Table 4 shows that antiserum, given i.p. on day 3 to mice which had been irradiated before inoculation with a small virus dose, gave considerable protection. Approximately 66% of these mice survived whilst none of the non-immune controls survived. As in the unirradiated mice which had been given post-infection ascitic fluid, clinical involvement when it did occur appeared late between the sixteenth and eighteenth days, compared to days 10–12 in the controls. This difference is reflected in the ADD shown in Table 4.

Kundin's experiments (1966) with an attenuated strain of Venezuelan equine encephalomyelitis virus indicate that there is a whole day's delay in the start of viraemia and an increase in virus titre following X-irradiation. As already stated there is evidence that the first phase of multiplication of many arboviruses may take place in the regional lymph node. If this is true for Langat virus in the mouse then the destruction of the lymphoid tissue by irradiation would deny the virus its first site for multiplication, lead to inefficient dissemination throughout the body and hence to delayed viraemia. Malkova (1962) using tick-borne encephalitis virus showed that this effect was most evident when a small virus inoculum was used. Similarly we found that, with a dose of 100 ICLD 50, viraemia was first detectable on the third day after inoculation. However, when viraemia was detectable on the

day after infection (Fig. 1) the inoculum was 20,000 times larger than that given in Table 4. It therefore seems probable that the administration of antibody on either of the first 2 days, and perhaps also on the third day, might completely prevent the establishment of infection in at least a proportion of these mice. This is further supported by the total absence of any histological changes, including neuronal damage, in six of the nine mice examined on the twelfth day. However, when infection does become established in such mice the infection behaves as in unirradiated mice which have been given antiserum, with a late onset of paralysis.

In conclusion, we believe that the CNS is probably always involved to some extent in any generalized virus infection with an encephalitis virus (and perhaps with any virus as almost all have caused an occasional case of encephalitis) and that neuronal damage is caused both by virus multiplication in the neurones and by the inflammatory changes with their associated oedema and hypoxia. Except perhaps in the very highly susceptible (usually very young) animals, neuronal damage due to virus multiplication alone is probably seldom sufficient to cause severe paralysis, coma or death, although lesser signs and symptoms result. However, severe clinical illness and death may result when secondary neuronal damage due to inflammation is superimposed: the degree of recovery so common, for instance, in poliomyelitis when the patient survives the acute stage, is excellent evidence of how much of the dysfunction is attributable to inflammatory overlay. If acceptable means can be found to control the immunological responses and suppress the inflammatory aspect of these diseases, considerable amelioration of the clinical illness might result.

SUMMARY

1. Irradiation in a whole body dose of 200 rads or more increased the sensitivity of mice to intraperitoneal infection with Langat virus so that the LD 50 was increased to about the intracerebral LD 50.

2. In mice given 500 rads before infection: (*a*) viraemia was prolonged by about 5 days; (*b*) the IgM response was depressed; (*c*) the IgG response was delayed by about 3 days and depressed in titre; (*d*) virus concentration in the brain rose continuously until death on about the tenth day while in the controls it reached a peak on the fifth day then subsided; (*e*) histological changes in the CNS were delayed and minimal even at death; (*f*) irradiated mice died with little evidence of paralysis while the controls died with severe paralysis.

3. In irradiated mice, protection was observed when antibody was administered on the third day following infection. Antibody given on the 3 days after infection to control mice aggravated the disease.

4. The results in this and the preceding paper are discussed in relation to the pathogenesis of encephalitis. It is concluded that neuronal damage is caused both by virus multiplication in neurones and by damage superimposed by inflammatory changes with associated oedema and hypoxia. The inflammatory changes appear to be due to an allergic reaction to virus-antibody complexes formed in the circulation and in the central nervous system.

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