The Pathogenesis of Avirulent Semliki Forest Virus Infections in Athymic Nude Mice

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SUMMARY
The course and outcome of intraperitoneally induced infections with the avirulent strain A7(74) of Semliki Forest virus have been studied in athymic ‘nude’ (nu/nu) mice, their heterozygous (nu/+) littermates and conventional Swiss A2G mice. The main distinguishing characteristics of the infection in the nu/nu mice were the persistence of virus in the brain after an initial phase of incomplete virus clearance and the apparent establishment of a secondary phase of virus replication in the brain which was associated with a falling neutralizing antibody response. This secondary phase of virus replication persisted until at least the 28th day after inoculation. In addition the typical histological lesions of encephalitis induced by this virus were rare and focal demyelination, which occurred at a light microscopy level in up to 26% of nu/+ and Swiss A2G mice, was not observed. It is suggested that in immunocompetent mice the development of lesions including demyelination may be a result of an immunopathological response to virus infection which is related to the presence of thymus derived lymphocytes.

INTRODUCTION
The ‘nude’ (nu/nu) mouse mutant was first described by Flanagan (1966) and later shown to be athymic by Pantelouris (1968). Most functional tests suggest that these mice are severely, if not entirely, devoid of thymus-dependent (T) lymphocytes and as a result T cell-dependent immunological responses are severely impaired (Wortis, 1971; Pritchard & Micklem, 1974). Until recently, investigations into the role of thymus-dependent immunological responses in the pathogenesis of virus infections have had to rely on the use of antithymocyte serum (ATS) or more complex procedures involving surgical thymectomy, lethal irradiation and bone marrow reconstitution. The T cell depletion produced by these methods is usually shortlived or incomplete and with the wider availability of nu/nu mice the use of these animals is gaining acceptance as a more reliable alternative. Previous experiments with SFV A7(74) in conventional Swiss A2G mice given ATS showed only marginal alterations in the course and outcome of the infection and no increase in mortality (Suckling et al. 1977). Experiments by Bradish et al. (1975) using cyclophosphamide administered up to 3 days before SFV A7(74) in conventional Porton mice also found little alteration in viraemia or antibody levels but there was a significant increase in mortality to approx. 50%. They attributed this effect to the inhibitory action of the drug on proliferating T cells rather than to its well established selective suppression of B cells (Turk, 1972). The experiments described here were designed to investigate the course and outcome of SFV A7(74) infections in athymic nu/nu mice as compared to their heterozygous nu/+ littermates and
conventional Swiss $A_2G$ mice and help to determine the extent to which T cell mediated immunological responses were involved in the pathogenesis of the disease.

METHODS

Mice. A breeding nucleus of BALB/c/CBA mice carrying the nude (nu) gene was obtained from Dr L. Owen of the School of Veterinary Medicine, University of Cambridge. The Cambridge colony was developed by random cross breeding of two carrier strains, BALB/c and CBA, originally obtained from the Laboratory Animals Centre, Carshalton, Surrey. Homozygous (nu/nu) mice and their heterozygous (nu/+ ) littermates were bred in the Animal House of St Thomas' Hospital Medical School by mating male nu/nu mice with female nu/+ mice on a random basis. The mice were maintained under barrier conditions in conventional autoclaved cages using sterilized bedding, with sterilized food and water supplied ad libitum. Conventional random bred Swiss $A_2G$ mice, raised under similar conditions, were also used.

Virus. The Semliki Forest virus (SFV) used in these experiments was the avirulent strain A7(74) originally isolated by Bradish et al. (1971). Ampoules of freeze-dried culture medium containing the virus which had been passed twice in chick embryo cells (SFV A7[74], C2) were obtained from Dr C. J. Bradish of the Microbiological Research Establishment, Porton Down, Salisbury. On reconstitution, a pool of stock virus suspension was made up in sterile 0.75% bovine albumin phosphate saline (BAPS) at pH 7.0 and stored in sealed glass vials at -70 °C until use.

Assay of virus infectivity. The virus infectivity of a sample was determined by preparing serial 10-fold dilutions in sterile BAPS and inoculating an appropriate range of dilutions intracerebrally (i.c.), 0.02 ml, into groups of 4 to 6 suckling mice (Swiss $A_2G$) 2 to 4 days old. The i.c. 50% lethal dose (LD$_{50}$)/0.02 ml was calculated by the method of Reed & Muench (1938).

Experimental design. Groups of 4- to 5-week-old nu/nu, nu/+ and Swiss $A_2G$ mice of either sex were inoculated intraperitoneally (i.p.) with 0.1 ml of a 10$^{-4}$ dilution of stock virus suspension. This standard dose was equivalent to 10$^{4.0}$ suckling mouse i.c. LD$_{50}$. Several groups of mice were used to obtain a comprehensive series of samples but any variation between successive groups was minimized as far as possible by keeping the age of mice and dose of virus constant. Experiments were designed so that there was some overlap in the days selected for sampling between successive groups as follows (individual groups are shown within parentheses): days post-inoculation (days p.i.) (1, 2, 3); (3, 4, 5, 6); (6, 8, 10); (4, 7, 9, 11); (10, 11, 12, 13, 14, 17); (12, 13, 19, 20); (15, 16, 17); (14, 21, 28). Additional single groups of mice were also used for further histological and serological studies.

Sampling. Up to 4 nu/nu, nu/+ and Swiss $A_2G$ mice/day were killed by exsanguination under ether anaesthesia on the days indicated. If required, a sample of whole blood for virus assay was diluted immediately in sterile BAPS to give a 10% suspension and the remaining blood was retained for serum separation. Brains were removed whole then divided sagittally. Half brains for virus assay, together with the blood suspensions, were stored at -70 °C until titration. Immediately before titration 10% homogenates of brain in sterile BAPS were prepared.

Histology. Half brains for histological examination were stored in 10% formol saline prior to processing. Standard procedures were used in which 5 μm sections were stained with haematoxylin and eosin for conventional histology, or with Luxol fast blue/cresyl violet for the detection of demyelination.
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**Antibody assay.** Individual or pooled sera were stored at \(-20^\circ\)C prior to testing. Tests for haemagglutination-inhibiting (HAI) antibody employed the micro-methods described by Sever (1962). Antigens were prepared by the sucrose-acetone method (Clarke & Casals, 1958) and by protamine sulphate precipitation (Warren et al., 1949). Neutralizing antibody was detected using the baby mouse neutralization test as follows: equal volumes of heat-inactivated (56 °C for 30 min) test or control normal mouse serum were added to the same volume of stock virus suspension so that the mixture contained approx. \(10^6\) LD\(_{50}\)/ml. After incubation for 1 h at 37 °C the LD\(_{50}\) of each sample was determined by titration as described above using 2- to 4-day-old Swiss A\(_2\)G mice inoculated i.p. with 0.05 ml. The serum neutralization index (SNI), expressed in \(\log_{10}\), was taken as the difference between the i.p. LD\(_{50}\) of the test serum and that of the control normal mouse serum. Tests for HAI and neutralizing antibody were carried out on pooled sera from 2 to 4 mice unless stated otherwise.

**RESULTS**

**Clinical observations**

Daily inspection of all the experimental mice confirmed the original report by Bradish et al. (1971) that, in the vast majority of animals, SFV A7(74) infections are entirely subclinical. Where clinical signs of disease were present they seldom amounted to more than slight weakness of the hind limbs and general lethargy. Deaths were extremely rare in all three groups of mice and tended to occur suddenly between 7 and 14 days p.i. There were no apparent differences between the groups of mice in either the incidence of clinical signs of disease or deaths. Intercurrent bacterial infections, to which nu/nu mice are particularly susceptible, were not observed in any of the mice.

**Virus recovery from blood and brain**

Fig. 1 shows the virus infectivity of the blood and brain samples taken from the three groups of mice. It can be seen that virus replication in the nu/nu mice followed a distinct pattern which was markedly different from those of the nu/+ or Swiss A\(_2\)G mice.

Considering the course of the viraemia first, the only apparent difference between the groups was that while peak virus infectivity, averaging \(10^6.4\) i.c. LD\(_{50}\)/0.02 ml, was attained at 2 days p.i. in the nu/+ and Swiss A\(_2\)G mice, only marginally lower peak infectivity was attained 24 h earlier in the nu/nu mice and was already falling by 2 days p.i. Virus clearance from the blood of each group of mice was completed by 4 days p.i. when all samples tested were negative, and samples tested at intervals up to 28 days p.i. were also negative.

Although the differences in the course of the viraemia in the three groups of mice were relatively minor, differences in the course of brain virus replication were more marked. Virus infectivity in the brains of the Swiss A\(_2\)G mice rose rapidly to peak levels, averaging approx. \(10^6.9\) i.c. LD\(_{50}\)/0.02 ml at 3 days p.i. which were maintained until 7 days p.i. By day 8 p.i. infectivity had fallen to an average of \(10^5.5\) i.c. LD\(_{50}\)/0.02 ml and from 9 days p.i. onwards only occasional brains had traces of virus, with all samples negative from day 12 p.i. onwards. Virus infectivity in the brains of the nu/+ mice showed a slightly different pattern to that of the Swiss A\(_2\)G mice, rising more slowly to similar peak levels on days 5 and 6 p.i. Virus clearance also began a day earlier in these mice, with infectivity on day 7 p.i. already well below that of the Swiss A\(_2\)G mice, and all samples were negative from 11 days p.i. onwards.

In the nu/nu mice, brain virus replication initially followed a pattern which was closer to that of the Swiss A\(_2\)G mice than to that of their nu/+ littermates. Virus infectivity rose
Fig. 1. Virus infectivity in blood and brain and serum neutralization index of nu/nu, nu/+ and Swiss A2G mice inoculated i.p. with SFV A7(74). , nu/nu blood; , nu/nu brain; , nu/+ blood; , nu/+ brain; , Swiss A2G blood; , Swiss A2G brain. Virus infectivity is expressed as log_{10} suckling mouse i.c. LD_{50}/0.02 ml and symbols marked ~ indicate an infectivity < the value indicated. Columns (■) indicate SNI expressed in log_{10}. Arrows (↑), SNI > the level indicated.

rapidly to a slightly higher peak, averaging 10^{6.5} i.c. LD_{50}/0.02 ml on day 3 p.i. and in most cases remained at a high level ≥ 10^{6.5} i.c. LD_{50}/0.02 ml until 8 days p.i., although infectivity was falling slowly throughout this period. After 8 days p.i. infectivity fell more rapidly and by day 10 p.i. averaged 10^{5.5} i.c. LD_{50}/0.02 ml. However, final clearance of virus from the brain was not achieved and virus infectivity remained at trace levels or above, averaging 10^{5.5-5.5} i.c. LD_{50}/0.02 ml, in all but three brains up to 19 days p.i. By day 20 p.i. infectivity had begun to rise again and had reached a new peak level, averaging approx. 10^{5.0} i.c. LD_{50}/0.02 ml, by 21 days p.i., which was maintained on day 28 p.i. Isolated brain
samples showed signs of a rise in infectivity slightly earlier, notably a single remaining brain from one experimental group taken on day 17 p.i. which had an infectivity of $10^{4.1}$ i.c. LD$_{50}$/0.02 ml.

**Development of neutralizing and haemagglutination-inhibiting antibody**

Sera were first tested for neutralizing antibody on day 4 p.i. when the viraemia had terminated and then at appropriate intervals throughout the sampling period. The SNI of representative pooled samples from each group are shown in Fig. 1.

Neutralizing antibody development in the Swiss A$_2$G and nu/+ mice rose to maximal levels by 6 (Swiss A$_2$G mice) or 8 days p.i. (nu/+ mice) which were then maintained. In contrast, in the nu/nu mice, neutralizing antibody initially appeared to develop slightly faster than in the nu/+ mice but the maximal levels to be attained in the nu/+ or Swiss A$_2$G mice were never reached and the SNI remained between 3.5 and 4.1 until 19 days p.i. From 20 days p.i., as brain virus infectivity began to rise, there was a corresponding fall in SNI to 0.9 on day 28 p.i. It was interesting that serum from the single mouse with a high brain virus titre on day 17 p.i. also had a low SNI of 1.1.

Sera from each group of mice were also tested at appropriate intervals for the presence of HAI antibody. In both the nu/+ and Swiss A$_2$G mice sera tested on day 10 p.i. or earlier had titres of < 10 HAI units, and were considered to be negative, but from day 11 p.i. onwards positive titres of > 20 HAI units were recorded. All the sera from the nu/nu mice had titres of < 10 HAI units.

A second group of sera were tested individually for HAI antibody on days 10 and 28 p.i. In this experiment one sample out of four taken from nu/nu mice on day 10 had a titre of 10 while the remaining sera taken on days 10 and 28 p.i. all had titres of < 10 HAI units. A second i.p. dose of virus given on day 7 p.i. resulted in four out of four sera having titres of 10 on day 10 p.i. but titres on day 28 p.i. were still < 10 HAI units. The equivalent groups of nu/+ and Swiss A$_2$G mice given a single inoculation of virus had HAI titres of 20 to 40 (nu/+ ) or 20 to 80 (Swiss A$_2$G) on day 10 p.i. and 40 to 160 (nu/+ ) or 80 to 320 (Swiss A$_2$G) on day 28 p.i. These titres were not altered significantly after a second inoculation in either the nu/+ or Swiss A$_2$G mice.

**Histology**

Examination of representative brain sections from the Swiss A$_2$G mice showed histopathological changes which conformed to the previously described pattern of a mild encephalitis characterized by perivascular cuffing, microglial proliferation and astrocyte hypertrophy and hyperplasia (Chew-Lim, 1975) with focal microcystic (spongiform) lesions occurring in the white matter of the cerebellum, the mid-brain and brain stem (Mackenzie et al. 1978). In addition, foci of demyelination in the cerebellar white matter, first described after two or three successive inoculations of virus (Chew-Lim et al. 1977) but later found to occur in 25% of mice following a single inoculation (Suckling et al. 1978), were seen.

Lesions in the nu/+ mice were similar to those seen in the Swiss A$_2$G mice. Of a total of 35 brains examined from mice sampled between days 10 and 28 p.i., microcystic and inflammatory lesions were present in 33 (94%). This was comparable with the 95% incidence established for Swiss A$_2$G mice (Suckling et al. 1978) although the lesions appeared to be slightly more extensive. As in the Swiss A$_2$G mice focal demyelination was not seen on day 10 p.i. but a total of 7 (26%) out of the remaining 27 brains showed evidence of focal
demyelination, again at a comparable incidence to the Swiss A2G mice. The incidence of myelin damage in both nu/+ and Swiss A2G mice may prove to be considerably higher on electron microscopic examination and these studies are being carried out at present.

By contrast, in the nu/nu mice, characteristic lesions of SFV A7(74) encephalitis were extremely rare. Of a total of 50 brains examined from comparable groups of mice sampled between day 10 and 28 p.i. only 15 (30%) showed mild microcystic lesions and there was no evidence of any significant perivascular cuffing. In addition none of the 38 brains examined between day 14 and 28 p.i. showed any evidence of demyelination.

**DISCUSSION**

Following i.p. inoculation with SFV A7(74) there were marked differences in the course and outcome of the infection in the nu/nu mice as compared to the nu/+ and Swiss A2G mice. Although the viraemia was cleared normally in the nu/nu mice, the onset of brain virus clearance was delayed by 1 to 2 days as compared to the Swiss A2G and nu/+ mice. Initially brain virus clearance appeared to follow a similar pattern in each group of mice but total clearance was rarely achieved in the nu/nu mice and, after an interval of several days during which virus persisted in the brain at a consistently low level, the infectivity began to rise again reaching a new peak infectivity on day 21 p.i. which was maintained on day 28 p.i. These results were based on infectivity assays on samples from only 2 to 6 mice/sampling point but it seems unlikely that either the period of consistently low virus infectivity or the high virus infectivity on days 21 and 28 p.i. occurred purely by chance, particularly as out of four mice from the same experimental group sampled on day 14 p.i., three had infectivities of $10^{5}$, $10^{2.5}$ and $10^{2.5}$ i.c. $LD_{50} / 0.02$ ml and in one mouse, virus was not detected. Taken with the evidence of low infectivity before rising infectivity in other sampling groups it would appear that a secondary phase of virus replication occurred in the brains of the nu/nu mice. As there was no evidence of a recurrence of the viraemia during this secondary phase of virus replication and the SNI was falling, any re-stimulation of the peripheral immunological system, which might initiate a second attempt at virus clearance, seems unlikely. It therefore appears that a persistent, subclinical CNS virus infection was established in the nu/nu mice.

In spite of the prolonged virus replication in the brains of the nu/nu mice only minimal histopathological changes were induced. There was no obvious cellular damage caused by the virus and both spongiform lesions and perivascular cuffing were either extremely mild or absent. In addition there was no evidence of demyelination in any of the nu/nu brains. This suggests that there was an immunopathological basis to the development of these lesions in the immunocompetent nu/+ and Swiss A2G mice, which was directly or indirectly dependent on the presence of competent T lymphocytes.

In this respect there are certain similarities to lymphocytic choriomeningitis (LCM) virus infections of mice. Lymphocytic choriomeningitis is the classical example of an immunopathological disease and immunocompetent mice inoculated i.c. with LCM virus develop an acutely fatal infection while immunosuppressed mice develop a non-fatal, persistent infection with minimal or absent histological lesions. It has been shown that the production of classical LCM is directly dependent on the presence of competent T lymphocytes (Cole et al. 1972). As would be expected nu/nu mice survive LCM virus infection. Unlike SFV A7(74), LCM virus did not induce a detectable antibody response and virus persisted in the blood with no evidence of an initial clearance phase although, after an early peak, the virus infectivity did stabilize at a slightly lower level (Christoffersen et al. 1976). Brain virus
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infectivity was not recorded but in a similar experiment by Mori et al. (1976) there was evidence that a persistent CNS virus infection had been established.

It remains to be seen whether any long term histopathological changes will occur in the brains of nu/nu mice as a result of persistent virus replication, or whether any clinical signs of disease will develop. In conventional Porton mice infected with the related avirulent A8 strain of SFV advanced lesions of hydrocephalus, spongy degeneration and a severe astrocytic reaction were seen in mice two years after recovery from an acute infection. This chronic slow disease was not associated with a detectable persistent CNS virus infection (Zlotnik et al. 1972).

The other aspect of SFV A7(74) infection in nu/nu mice which was of particular interest concerned the failure of initial attempts at brain virus clearance. Previous studies with SFV A7(74) in conventional Swiss A$_4$G mice have suggested that normal antibody development plays an important part in virus clearance. In mice immunosuppressed with cyclophosphamid, which selectively depletes B lymphocytes (Turk, 1972), a single dose of the drug given 24 h after virus inoculation significantly prolonged virus replication in blood and brain accompanied by a marked delay in humoral HAI antibody development and a reduction in the intensity of perivascular cuffing (Suckling et al. 1977). Similarly, in conventional Porton mice infected with SFV A7(74), maximum suppression and slowest recovery of humoral antibody activity, associated with a prolonged viraemia and an increased susceptibility to challenge with the lethal L10 strain of SFV, were obtained when cyclophosphamid was given 1 to 2 days after virus, results consistent with the effects of the drug on B lymphocytes (Bradish et al. 1975). In contrast, in mice given ATS, which depletes T lymphocytes (Levey & Medawar, 1966), there was no detectable delay in the termination of the viraemia and brain virus replication was only slightly prolonged with, at most, only a marginal delay in humoral antibody production and no obvious reduction in the intensity of perivascular cuffing (Suckling et al. 1977). In the nu/nu mice the initial production of neutralizing antibody appeared to be relatively normal and the viraemia was terminated without delay. However from 8 days p.i. onwards the SNI was consistently below that of the nu/+ and Swiss A$_4$G mice and as the secondary phase of virus replication became established the SNI began to fall reaching an extremely low level by day 28 p.i. Traces of HAI antibody were detected occasionally on day 10 p.i. but this response was not maintained.

Transient production of antibody has been reported to occur following a number of virus infections in nu/nu mice but sustained antibody responses to all the viruses tested were markedly depressed or absent (Burns et al. 1975). In the case of Sindbis virus, an alphatogavirus related to SFV, neutralizing antibody production was detectable for 2 weeks and shown to be solely IgM. Normal nu/+ littermates initially produced IgM but were also capable of mounting a sustained IgG antibody response. The authors concluded that IgM production to virus protein antigens is relatively thymus independent while the switch from IgM production to sustained IgG production is highly thymus dependent.

While a predominantly IgM antibody response would be capable of neutralizing virus in the blood and hence terminating the viraemia, IgM is unlikely to cross the blood-brain barrier particularly in the absence of severe inflammation. A recent paper by Fleming (1977) on the antibody response to SFV A7(74) in conventional Porton mice has shown that IgM was only a minimal component in the brain, where the onset of virus clearance coincided with the appearance of IgG$_3$, followed by IgG$_2$. Although specific IgG synthesis is severely impaired in nu/nu mice the response is not completely abrogated (Pritchard & Micklem, 1974) and on the basis of these results it seems likely that some IgG production, perhaps only IgG$_3$, may have occurred in the nu/nu mice. If there was then a failure to
switch to sustained IgG production, probably IgGα, the antibody response would terminate following the natural decline in IgM production. Thus, although brain virus clearance could be initiated, the antibody response would not be sustained for long enough to enable total virus clearance to occur allowing the virus to ‘escape’ and enter a secondary phase of virus replication.

In conclusion, infections of nu/nu mice with SFV A7(74) clearly merit more extensive investigation, including a detailed study of the exact nature of the antibody response, which should provide further insight into the pathogenesis of the disease. In addition use of the SFV A7(74) nu/nu mouse model should assist indirectly in determining the mechanisms involved in the production of demyelination in immunocompetent mice.

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