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Immunoglobulin synthesis in nude (nu/nu), nu/+ and reconstituted nu/nu mice infected with a demyelinating strain of *Semliki Forest virus*

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SUMMARY

Infections with the avirulent (A7/74) strain of *Semliki Forest virus* which causes primary demyelination of the central nervous system in mice have been studied further in nude athymic (nu/nu) mice and their immunocompetent (nu/+) litter mates to measure the production of immunoglobulins. This has been done by radial immunodiffusion and enzyme-linked immunosorbent assays. Half the nude mice examined were able to synthesize specific IgG but at levels 1,000-fold lower than their nu/+ littermates. The majority of nude mice reconstituted with spleen cells from nu/+ mice 1 day before infection with virus were able to synthesize specific IgG nearly as well as the nu/+ animals.

INTRODUCTION

The ability of nude athymic (nu/nu) mice to synthesize immunoglobulins in response to foreign antigens is known to vary according to the degree of thymus dependence or independence of the antigen. In the case of bacterial polysaccharide antigens, which are highly thymus independent, the immunoglobulin response of nude mice is equal to or greater than normal, while the response to sheep red blood cells, which are highly thymus dependent, is grossly impaired particularly in respect of IgG (Pantelouris, 1978). The response to viral antigens has been examined by Burns, Billups & Notkins (1975) who demonstrated that although sustained IgG synthesis was markedly thymus dependent, nude mice were capable of mounting an early but transient IgM response of normal magnitude to a number of viruses, including the *Togavirus sindbis*. Previous studies in nude mice infected with *SFV* A7(74) have shown that neutralizing antibodies are initially produced at a level similar to that seen in the immunocompetent nu/+ mice, but declines to low levels as the infection progresses (Jagelman *et al.*, 1978). This observation has been confirmed in an independent study which also demonstrated that IgM was synthesized as the dominant Ig class (Bradish *et al.*, 1979). In the strain of nu/nu mice these authors used, which had a different derivation from those used by us, they found histological lesions in the brains of nu/nu and nu/+ mice similar to those found in the *Swiss* A_2G immunocompetent mice inoculated with the same strain of *SFV*. This was in contrast to our studies in which the neuropathological lesions, including demyelination normally seen in our immunocompetent *Swiss* A_2G mice, were extremely rare in the strain of nu/nu mice we used, in spite of high brain virus titres persisting for 28 days (Jagelman *et al.*, 1978). The apparent deficiency in IgG synthesis in nude mice is of particular interest in the case of *SFV* A7(74) infections in view of the

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association of IgG3 with brain virus clearance and the possible immunopathogenic role of IgG2a (Fleming, 1977). We have therefore attempted to determine the extent of any IgG synthesis by our strain of nu/nu mice in response to *SFV* A7(74) infection as compared to their heterozygous nu/+ littermates and to spleen cell reconstituted nu/nu mice in the hope that it will help us to understand the pathogenesis of the demyelination.

MATERIALS AND METHODS

Mice. A breeding nucleus of BALB/C-CBA mice carrying the nude (nu) gene was obtained from a colony originating from the Laboratory Animals Centre, Carshalton, Surrey but maintained at the Imperial Cancer Research Fund, Mill Hill, London. 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*. Animals were used for experiments at 4–6 weeks-of-age.

Reconstitution of nude mice. Nu/+ littermates of either sex were killed under ether anaesthesia and their spleens removed aseptically. The spleen cells were disaggregated by rubbing through sterile stainless steel gauze (mesh 23/cm) into cold medium 199, containing 5% fetal calf serum, 600 μg/ml penicillin and 1,000 μg/ml streptomycin, followed by gentle Pasteur pipetting. The pooled cell suspension was allowed to stand for approximately 1 min and the supernatant was centrifuged at 100 ***g*** for 10 min at 4°C. The cells were resuspended in medium 199 prior to inoculation intraperitoneally (i.p.) in volumes of 0·3–0·6 ml. Each recipient mouse received approximately 10^8 viable cells (equivalent to the number of cells received from each donor mouse) as assessed by the Trypan blue exclusion test (viability ± 95%). Reconstitution was carried out 24 hr prior to virus inoculation.

Virus. The *Semliki Forest* virus (*SFV*) used in these experiments was the avirulent strain A7(74) originally isolated by Bradish *et al.*, (1971). Culture medium containing the virus, which had been passed twice in chick embryo cells (SFV A7(74).C2), was obtained from Dr C. J. Bradish of the Microbiological Research Establishment, Porton Down, Wiltshire, England. A pool of stock virus suspension was made up in sterile 0·75% bovine albumin phosphate saline (BAPS) at pH 7·0 and stored at −70°C until used. The virus pool had a titre of $10^{9\cdot1}$ $ICLD_{50}$/ml in baby mice. Experimental mice were inoculated i.p. with 0·1 ml of virus suspension containing $10^{4\cdot1}$ $ICLD_{50}$.

Single radial immunodiffusion (SRID). The SRID method used was essentially that of Mancini, Carbonara & Heremans (1965). Gel diffusion plates containing 1% of rabbit anti-mouse IgG (Miles) were prepared using 1·5% agarose (Indubiose A37) spread to standard depth on 7·5 × 4·0 cm glass slides. Wells in the slides (1·5 mm in depth, 3·0 mm in diameter) were filled with dilutions of test sera (20 μl). After incubation in a moist chamber for 48 hr at room temperature the plates were dried and stained with coomassie blue. The diameters of the stained precipitin rings were measured. Using a mouse IgG reference standard (Miles) a standard curve was constructed and allowed quantitation of test serum samples.

Double diffusion. The gel diffusion plates were prepared from 1·5% agarose spread on 7·5 × 4·0 cm glass slides into which a hexagonal well pattern was punched (well size, 1·5 mm in depth, 3·0 mm in diameter). Dilutions of test sera (20 μl) were placed in the centre well. Antisera were supplied by Miles Laboratories (rabbit anti-mouse IgG, IgM) and Nordic Immunological Laboratories, Maidenhead, Berkshire (rabbit anti-mouse IgG1, goat anti-mouse IgG2a, IgG2b, IgG3). After overnight incubation precipitin lines were stained as above. Results were expressed in terms of the highest dilution at which a precipitin line was observed.

Enzyme linked immunosorbent assay (ELISA). The ELISA technique used was based on the indirect method of Voller, Bidwell & Bartlett (1976). Antigen was purified from *SFV* A7(74) infected baby mouse brains using the method described by Faulkner & McGee-Russell (1968). A glutaraldehyde conjugate of alkaline phosphatase (Sigma type VII) and the IgG fraction of rabbit

antiserum to mouse IgG was prepared following the method described by Engvall & Perlmann (1972). The test was conducted as follows:—

Antigen diluted 1:500 in pH 9·6 carbonate coating buffer was added to each well of a Dynatech M129A polystyrene microelisa plate in aliquots of 200 μl and incubated overnight at 4°C. After washing each well three times with 200 μl 0·1 M PBS (pH 7·4) containing Tween 20 at 0·5%, 200 μl of test sera, diluted from 10^{-1} to 10^{-6} in PBS-Tween, were added to appropriate wells and incubated at room temperature for 2 hr. The wells were then washed in PBS-Tween as before, prior to the addition of 200 μl of conjugate diluted 1:150 in PBS-Tween. Following 2 hr incubation at room temperature and further washing in PBS-Tween, 200 μl p-nitrophenol phosphate (Sigma 104), diluted to contain 1 mg ml^{-1} diethanolamine buffer (pH 9·8) was added to each well. The reaction was stopped after 30 min at room temperature by the addition of 50 μl of 3M NaOH and the absorbance of each well read at 400 nm.

Under our test conditions sera from uninoculated mice diluted 1:10 gave absorbances at 400 nm of $<0{\cdot}25$, as did sera from mice infected with other related or unrelated viruses. Test sera were considered positive when their absorbance at 400 nm exceeded 0·25 and results are expressed as the highest positive dilution. In addition, each plate contained a known positive serum to control plate to plate variation.

The CNS histology was examined in all animals and will be reported in detail later.

RESULTS

As in our previous study evidence of infection with perivascular cuffing and demyelination occurred in the nu/+ mice with little evidence of infection except for microcystic changes in the nu/nu mice (Jagelman *et al.*, 1978). Reconstitution of the nu/nu mice produced changes similar to that in the nu/+ mice and included demyelination.

Total serum IgG levels

The total IgG levels estimated non-specifically by SRID are shown in Fig. 1. A sharp rise in IgG levels occurred in both the nu/+ and reconstituted nu/nu mice 10–12 days after inoculation and these high levels were maintained for at least 42 days post inoculation. Very little change occurred in

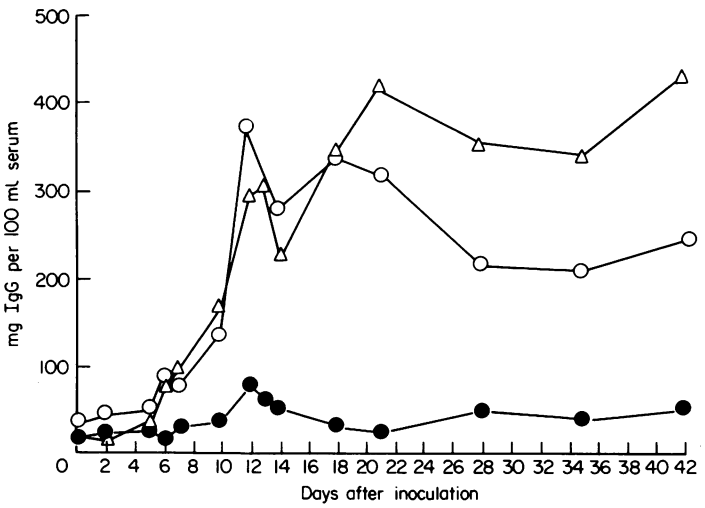

Fig. 1. Total serum IgG levels in nu/nu, nu/+ and reconstituted nu/nu mice after i.p. infection with *SFV* A7(74) estimated by radial immunodiffusion. (Values given as means of 2–7 individual mice.) (● = nu/nu mice; ○ = nu/+ mice; △ = reconstituted nu/nu mice.)

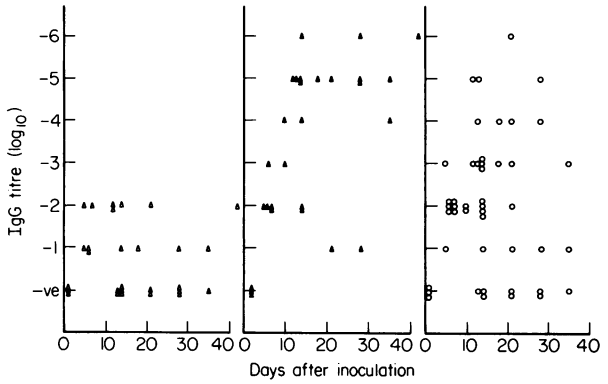

Fig. 2. Specific anti-*SFV* IgG titres in nu/nu, nu/+ and reconstituted nu/nu mice after infection with *SFV* A7(74) estimated by ELISA. Titres refer to the lowest dilution of serum at which a positive reaction was obtained. Each point represents an individual mouse. (△ = nu/nu mice; ▲ = nu/+ mice; ○ = reconstituted nu/nu mice.)

the nu/nu mouse IgG levels although there was a small, temporary, increase 12–14 days after inoculation, shadowing the rise seen in the nu/+ and reconstituted nu/nu mice.

It is known that serum IgG levels increase with age in immature mice up to about 10 weeks old (Bloemmen & Eyssen, 1973). As our mice were inoculated at 4–5 weeks of age it was necessary to determine that this change in IgG levels was not due to age alone or to a response to the virus diluent (BAPS). In mice inoculated with BAPS alone the IgG levels in nu/nu mice rose approximately 60% over a 4 week period from day 0, whilst levels in nu/+ animals rose approximately 240%. Both these increases were insignificant compared with those observed in infected mice. The observed increases in IgG levels cannot therefore be accounted for solely by an age related or BAPS induced response.

Specific anti-SFV IgG synthesis

In order to establish that the increases in total serum IgG, demonstrated by the SRID results described above, do represent a response to *SFV* A7(74) infection an ELISA technique was employed to assess specific anti-*SFV* IgG levels in the same groups of mice. The results are shown in Fig. 2. The nu/+ mice as expected were uniformly capable of synthesizing high titres of specific anti-*SFV* IgG from 5 days after infection. Perhaps surprisingly a proportion (54%) of the nu/nu mice were also able to produce specific IgG but only at a level about 1,000-fold less than in the nu/+ mice. The majority (82%) of reconstituted nu/nu mice were also able to synthesize specific IgG and of these, 40% reached levels above the highest detected in unreconstituted nu/nu mice.

IgM and IgG subclass synthesis

Although some IgG synthesis does occur in nu/nu mice, but at considerably reduced levels, as assessed by both non-specific (SRID) and specific (ELISA) techniques, we felt that it would be of interest to examine the extent to which synthesis of the IgG subclasses was affected. In addition the levels of IgM synthesis were examined. For the purposes of this preliminary assessment a non-specific double diffusion technique was used.

The results are shown in Fig. 3. It can be seen that levels of all the classes of Ig increased in the nu/nu mice following inoculation with *SFV* A7(74) but, with the exception of IgM, failed to reach the high levels seen in the nu/+ mice. IgM production was particularly efficient in the nu/nu mice with a rapid rise to peak levels by day 5, as compared to the slower rise to peak levels on day 10 seen in the nu/+ mice. In the case of IgG1 and IgG3 the increase in levels occurred at the same time in the nu/nu mice as in the immunocompetent nu/+ mice while the increases in IgG2a and IgG2b were delayed in the nu/nu mice. With the exception of IgG3 all classes of IgG and IgM were decreasing in level by day 49 in the nu/nu mice.

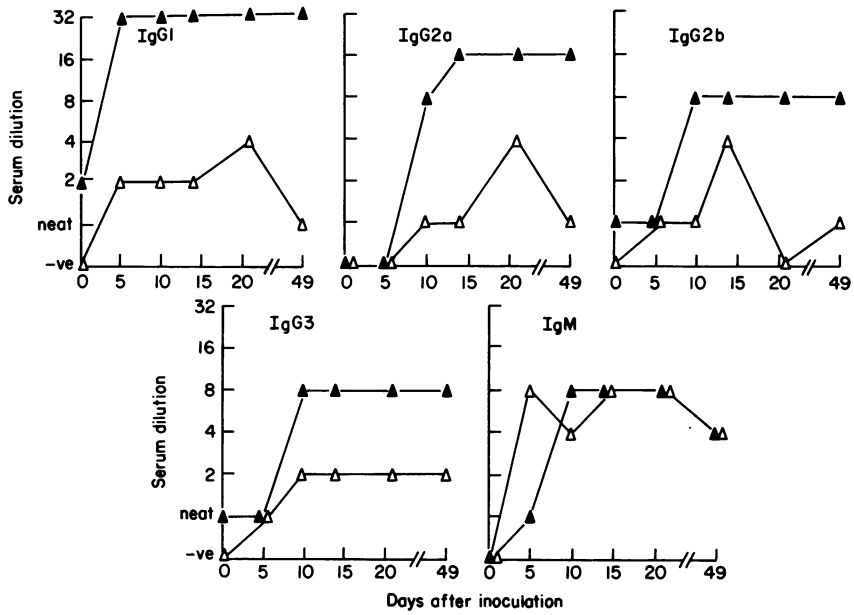

Fig. 3. IgG subclass levels in nu/nu and nu/+ mice after infection with *SFV* A7(74) determined by a double diffusion method. Points at day 0 indicate uninoculated values of 6 week old mice. All points represent an average value derived from separate mice on each sampling day. A negative value on the serum dilution axis indicates that no precipitin line was visible with undiluted serum. (△ = nu/nu mice; ▲ = nu/+ mice.)

DISCUSSION

A number of previous studies of immunoglobulin synthesis in nude mice have established that although serum levels of IgM are within, or sometimes above, the normal range, levels of IgG are usually significantly depressed (Pantelouris, 1978). Our initial results using the SRID technique to assess total serum IgG confirmed that levels in the control inoculated nu/nu mice were considerably lower than in their nu/+ littermates. However, following virus inoculation there was evidence of a transient increase in IgG levels. Further investigation, using the ELISA technique, established that approximately half of the nu/nu mice were in fact capable of synthesizing specific anti-*SFV* IgG, in some cases as early as 5–7 days after infection. Although the levels of IgG were low and showed no signs of rising during the course of the infection, these results strongly suggest that at least some specific IgG synthesis is thymus independent. This result was somewhat unexpected in view of previous studies with virus infections in nude mice (Burns *et al.*, 1975) but may simply reflect the high degree of sensitivity of the ELISA technique.

There is evidence to suggest that some classes of IgG are particularly deficient in nude mice. Bankhurst, Lambert & Miescher (1975) found that, as compared to nu/+ littermates, the levels of IgG1 in unstimulated nu/nu mice were reduced to 8% and of IgG2a to 31% while levels of IgG3 and IgG2b were not significantly altered. Our preliminary attempts at assessing the extent of IgG subclass synthesis following virus infection suggested that although there was some response in each case, IgG1 and IgG2a synthesis was most noticably affected and IgG3 synthesis least affected. Adaptation of the ELISA test to measure specific anti-*SFV* IgG subclasses would be possible and should clarify these results.

The other aspect of the work described in this paper concerns the ability of spleen cell transfer from immunocompetent nu/+ littermates to nu/nu mice to reconstitute their immunological responses. Spleen cell transfer was selected as this has been found to produce a better response, particularly in terms of antibody synthesis, than either thymus cells or whole thymus grafts

(Kindred, 1978). Our results confirm that in terms of total IgG, levels in reconstituted nu/nu mice infected with *SFV* A7(74) were restored to a similar level to that seen in the nu/+ mice. The synthesis of specific anti-*SFV* IgG was also increased in the majority of reconstituted mice reaching levels as high as those in the nu/+ mice in many cases.

Previous studies on *SFV* A7(74) infections in nude mice have shown that sustained antibody synthesis, brain virus clearance and the development of characteristic histopathological changes, are to a considerable extent thymus dependent (Jagelman *et al.*, 1978). The association of intracerebral immunoglobulins with brain virus clearance has already been established (Fleming, 1977), but their possible role in the production of immunopathological lesions is still unclear. The ELISA technique obviously has great potential for use in further detailed studies of specific IgG production in nu/nu mice and their immunocompetent nu/+ littermates. Together with the ability to reconstitute the antibody response by spleen cell transfer, applications of these techniques should be of considerable value in determining the exact role of thymus dependent and independent antibody responses in the pathogenesis of the disease and in particular their role in the production of the primary demyelination.

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