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Studies on the Immune Response and Pathogenesis of Sendai Virus Infection of Mice

III. THE EFFECTS OF CYCLOPHOSPHAMIDE

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Summary. Studies of the effects of cyclophosphamide on a non-lethal primary Sendai virus infection of mice are reported. Treatment with cyclophosphamide resulted in failure to limit or eradicate virus, diminished and delayed the appearance of immunoglobulin-secreting cells in the lungs and the production of local and serum antibody, reduced and delayed the appearance of bronchial basement membrane damage and the desquamation of infected mucosal cells, and reduced the incidence of immune complex deposition in the kidneys. Evidence is presented which indicates that some escape from immunosuppression occurred by day 6, resulting in local antibody production. The appearance of the local antibody response was associated with increased tissue damage in the lungs and the deposition of immune complexes of viral antigen and antibody in the kidney. Nine further experiments were performed in mice to investigate this renal manifestation and preliminary results are presented. In four of seven Sendai and one of two avirulent influenza A (Kunz) virus infections glomerular immune complexes were found. Studies in C3H and C57 Bl mice and their F1 hybrid suggested that genetic factors play some part in the renal findings.

The results are discussed with respect to the possible beneficial and harmful effects of the immune response to trivial respiratory virus infections.

INTRODUCTION

A well documented primary non-lethal Sendai virus infection of mice (Robinson, Cureton and Heath, 1968) was converted into a lethal infection when the animals were treated with cyclophosphamide (Robinson, Cureton and Heath, 1969). This was associated with failure to eradicate virus, normal interferon production, leukopenia, depression and delay of the mononuclear infiltration of the lungs, and absent serum antibody production. It has subsequently been shown with the same virus in normal animals that immunoglobulin (Ig) secreting cells appear in large numbers adjacent to infected bronchial mucosal cells as early as the 2nd day after infection (Blandford, Cureton and Heath, 1971) and that specific mouse anti-Sendai virus antibody is detectable in lung tissues by the 3rd day (Blandford and Heath, 1972). These data strongly suggest that virus eradication is brought about by local antibody production.

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The studies reported here set out to determine if local antibody was produced in the lungs of cyclophosphamide-treated mice. Traces of local antibody were found later than in normal animals when some escape from immunosuppression had occurred and was associated with damage to the bronchial basement membrane. This damage was not seen in the absence of local antibody. As a part of the experimental design, kidneys were obtained from all animal groups. Studies of this organ led to information which implicates the immunological response to respiratory virus infection as a potentially very important factor in the pathogenesis of acute, and possibly chronic, immunologically mediated renal disease.

MATERIALS AND METHODS

Experimental design

Four groups of female, random-bred, specific pathogen-free, Swiss white mice (25 g) were treated according to the following protocol. One group (S mice) were infected intranasally (i.n.) with approximately 10^5 EID_{50} Sendai virus (Robinson *et al.*, 1968) and given 0·2 ml of normal saline (NS) on days 1 and 5 after infection, intraperitoneally (i.p.). The second group (SC mice) were similarly infected and given cyclophosphamide (200 mg/kg) in 0·2 ml i.p. The third group (C mice) were given NS i.n. and cyclophosphamide according to the same schedule as SC mice. The fourth group (N mice) were given NS both i.n. and i.p. Six animals from each group were killed at day 0 and days 3, 6, and 9. Two repeat experiments were performed.

Lungs, spleens and kidneys were dissected from exsanguinated animals and prepared for studies of virus growth using 10 per cent w/v suspensions in culture medium (Robinson *et al.*, 1968). Tissues for immunohistological studies were fixed in cold alcohol (Sainte-Marie, 1962).

Virus assay

Cultured monkey kidney cells were exposed for 36 hours to various dilutions of homogenized lung, spleen or kidney tissue, as above, and virus growth was detected by haemagglutination (Robinson *et al.*, 1968).

Antibody assay

Serum antibody was detected by haemagglutination inhibition (Blandford *et al.*, 1971). Antibody present in tissues was detected by observing increased fluorescent staining for viral antigens after tissue sections had been eluted with acid buffer, pH 2·8, for 1 hour, or after 24 hours incubation with high titre rabbit anti-Sendai virus antibody (Blandford and Heath, 1972).

Fluorescence technique

The indirect immunofluorescence technique and antisera used in staining for viral antigens and mouse immunoglobulins were as previously reported (Blandford *et al.*, 1971; Blandford and Heath, 1972, 1974). Examination of sections was by a Leitz Orthoplan microscope equipped with the Ploem incident light illuminator. UGI excitation and K460 absorption filters with dark ground transmitted light were used for the lung studies and BG 12 excitation and K530 absorption with incident light were used for the renal studies. The latter revealed more positive staining than the former, as has been previously reported (Markham, Sutherland and Mardiney, 1973).

RESULTS

WHITE BLOOD COUNTS

The white blood cell counts (WBCC) in the four groups of animals are depicted in Fig. 1. It can be seen that those animals which received cyclophosphamide became

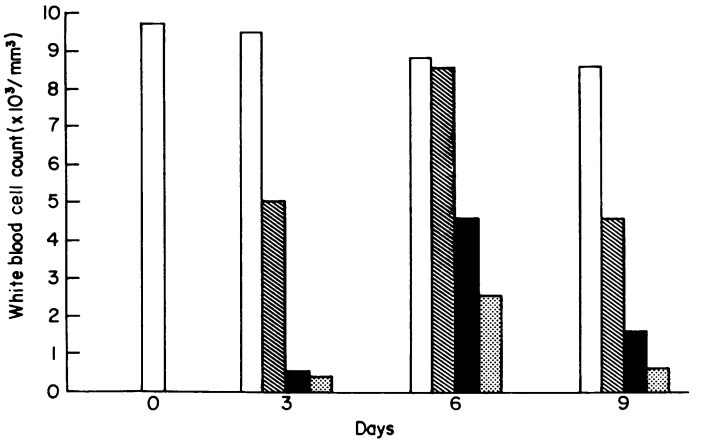

FIG. 1. Histogram showing mean WBCC of nine mice from each group on each experimental day. Cyclophosphamide was given on days 1 and 5. Blank columns saline controls. Hatched columns, Sendai virus. Solid columns, cyclophosphamide. Stippled columns, cyclophosphamide/Sendai virus.

profoundly leukopenic following the first dose and that there was an increase in the WBCC on the day after the second dose was given (day 5). At day 9, the WBCC were not quite as low as they had been at day 3. Sendai infection alone induced a lesser degree of leukopenia with WBCC reduced by about half at days 3 and 9. At day 6 an increase in the WBCC was again seen. SC mice showed more profound leucopenia than either S or C mice and once again an increase in the WBCC was seen at day 6. The rise in WBCC at 6 days in the C and SC animals would suggest that some escape from immunosuppression had occurred by the time the second dose of cyclophosphamide was given.

VIRUS GROWTH

No virus growth was obtained from the lungs of C or N mice or from the spleen or kidney of any group. In SC mice virus growth from the lungs continued up to and including the 9th day whereas in S mice replicating virus was eradicated by this time.

SERUM ANTIBODY TITRE

A serum haemagglutinating inhibiting antibody titre of at least 1:64 was found in all S mice by day 9. SC mice did not have a detectable serum antibody response. No antibody was detectable in C or N mice at any time.

DISTRIBUTION OF VIRAL ANTIGENS

Immunofluorescent studies of the lungs for viral antigens showed no virus in C or N mice. At day 3 (Figs 2 and 3) and day 6 more viral antigen was detectable in the bronchi

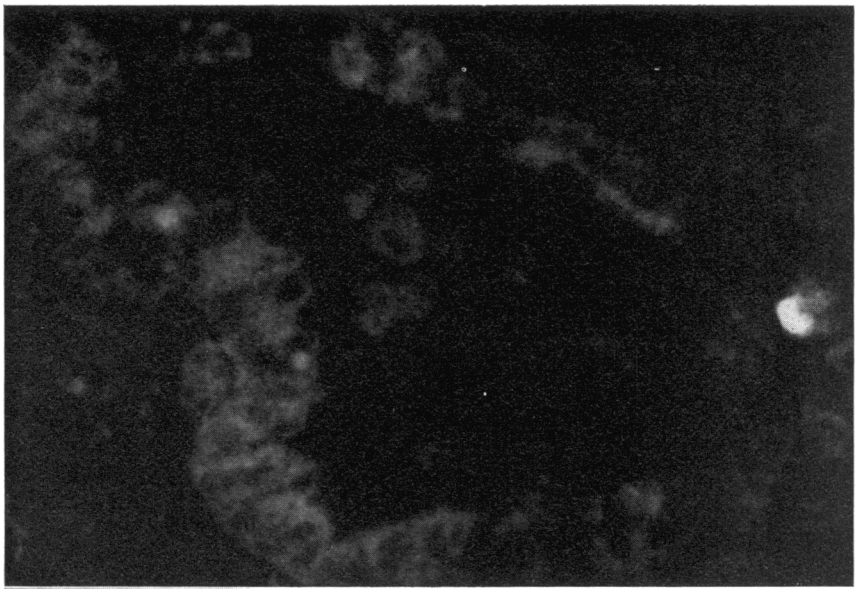

FIG. 2. Cross-section of bronchus from Sendai virus-infected mouse, day 3. Immunofluorescent staining for viral antigens. (Magnification × 1610.)

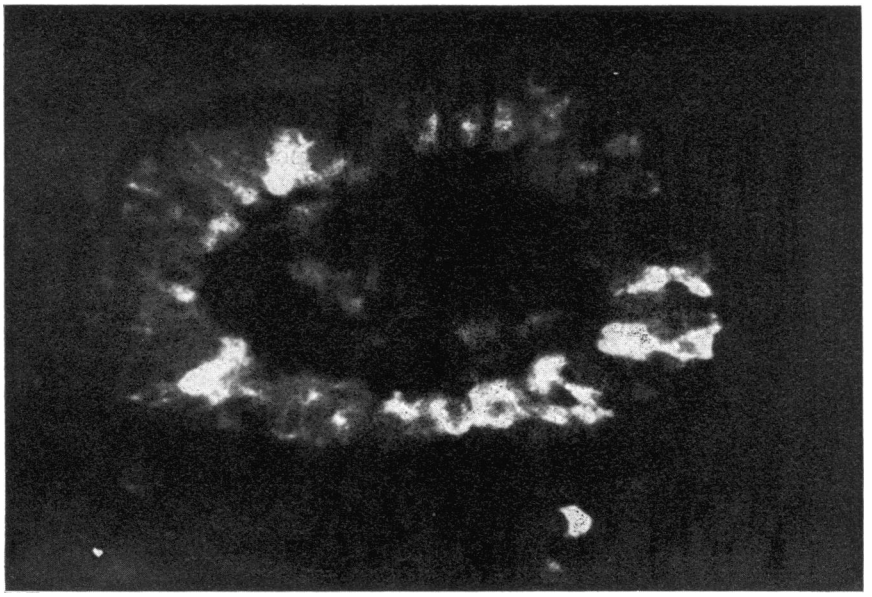

FIG. 3. Cross-section of bronchus from Sendai virus-infected, cyclophosphamide-treated mouse, day 3. Immunofluorescent staining for viral antigens. (Magnification × 1610.)

of SC mice than of S mice and it was apparent that virus had spread further and faster in the SC mice. The usual desquamation of infected cells seen at day 3 in S mice was not seen at this time in SC mice although many more bronchi were infected. SC mice showed same desquamation of infected mucosal cells at day 6. Sendai viral antigens were detected in alveolar cells and pneumonic areas in both S and SC mice, but were much more extensive and earlier in SC mice. At day 9 no viral antigens were found in S mice, but were still present in SC mice though in diminished amounts compared to the findings at day 6.

Elution studies of tissue sections at day 3 revealed more viral antigens in S mice but not in SC mice. At day 6, when some desquamation had occurred in SC mice, there was increased fluorescence for Sendai antigens in these areas after acid elution. At day 9 viral antigens were not found in S mice but some increased fluorescence after acid elution was noted in SC mice. This was present in pneumonic areas of lung as well as in the bronchi. These findings suggest an absence of local antibody in SC mice at day 3 but the presence of antibody at day 6 and day 9, confirming that some escape from immunosuppression had occurred.

CONVENTIONAL HISTOLOGICAL STUDIES

There were essentially no differences in the conventional histological studies of the lungs of N and C mice, although the latter took up stains less well. Changes similar to those previously reported occurred in the S and SC mice (Robinson *et al.*, 1968, 1969). Thus, few mononuclear cells appeared in the SC bronchial sub-mucosa, although some polymorphs were present. There was also less peribronchial and perivascular oedema in this group. A few mononuclear pyronin-positive cells had appeared by day 6 in SC mice and were perivascular and peribronchial in distribution, similar to, but quantitatively much less than, in S mice at day 3. SC mice showed extensive areas of pneumonic consolidation in the peripheral lung fields at day 6 and day 9, increasing with time. This was the major lung pathology in the animals which died. By day 9 the cellular infiltration in pneumonic areas in SC mice contained many pyknotic cells, some large mononuclear phagocytic cells, and some pyronin-positive plasma cells.

Additional differences between S and SC mice concerned changes to the bronchial basement membrane (BBM) which had not been previously noted. In S mice the BBM at day 3 was a swollen, fragmented, and reduplicated structure, infiltrated with inflammatory cells (Fig. 4). This was a very abnormal appearance compared to N mice (Fig. 5). In C mice the BBM was a normal and distinct structure but did not stain well. The most surprising finding was that the BBM beneath the heavily infected mucosal cells of SC mice at day 3 was somewhat thickened but otherwise normal (Fig. 6). By day 6, however, the BBM in SC mice was quite similar in appearance to S mice at day 3. S mice at day 6 were beginning to reorganize the BBM beneath the regenerating mucosal cells, but the structure was still generally incomplete, even by day 9. This lack of an intact boundary BBM beneath the areas of squamous metaplasia added greatly to an appearance suggesting lung tumour.

IMMUNOGLOBULIN-SECRETING CELLS

Immunofluorescent staining of mouse lungs for immunoglobulins showed the previously

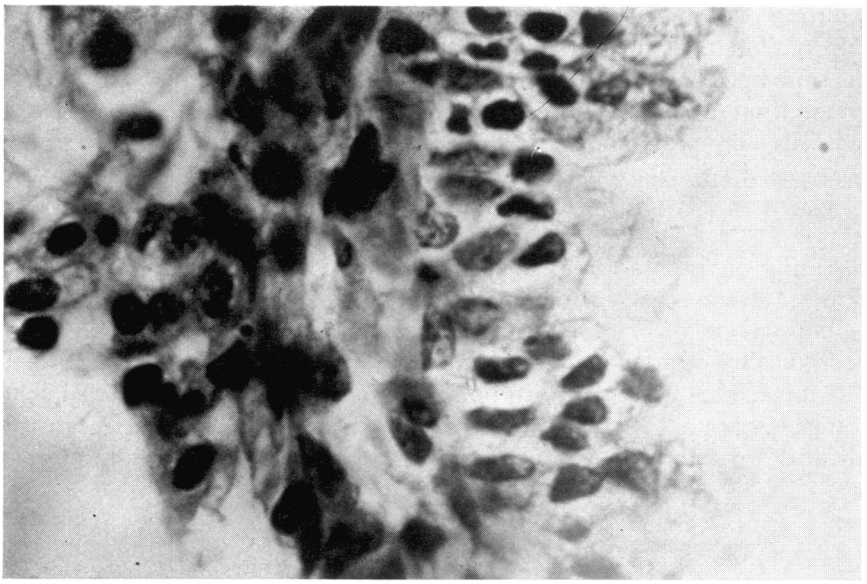

FIG. 4. Cross-section of bronchus from Sendai virus-infected mouse, day 3. Note the broad, indistinct, swollen and fragmented bronchial basement membrane and infiltration with inflammatory cells. (PAS; magnification × 4025.)

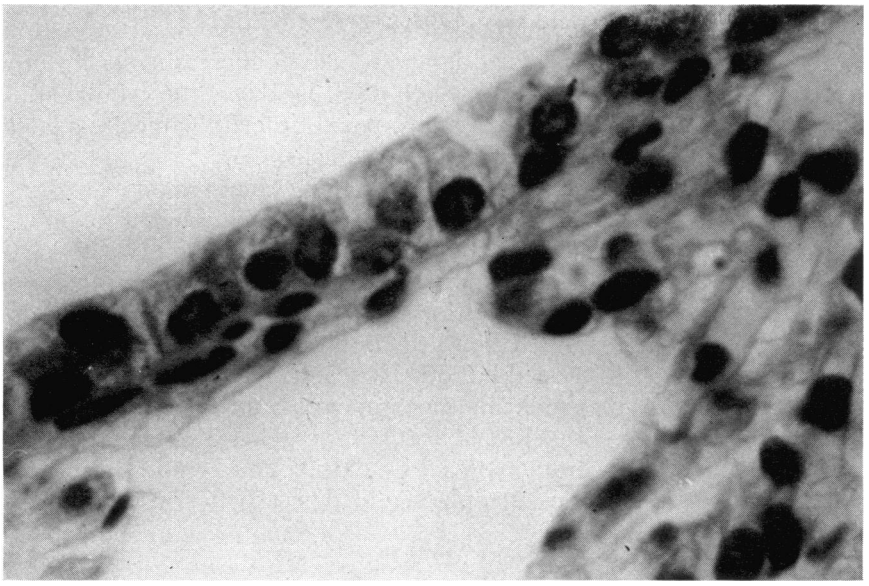

FIG. 5. Cross-section of bronchus from normal mouse. The bronchial basement is a fine structure defining the base of the mucosal cells. (PAS; magnification × 4025.)

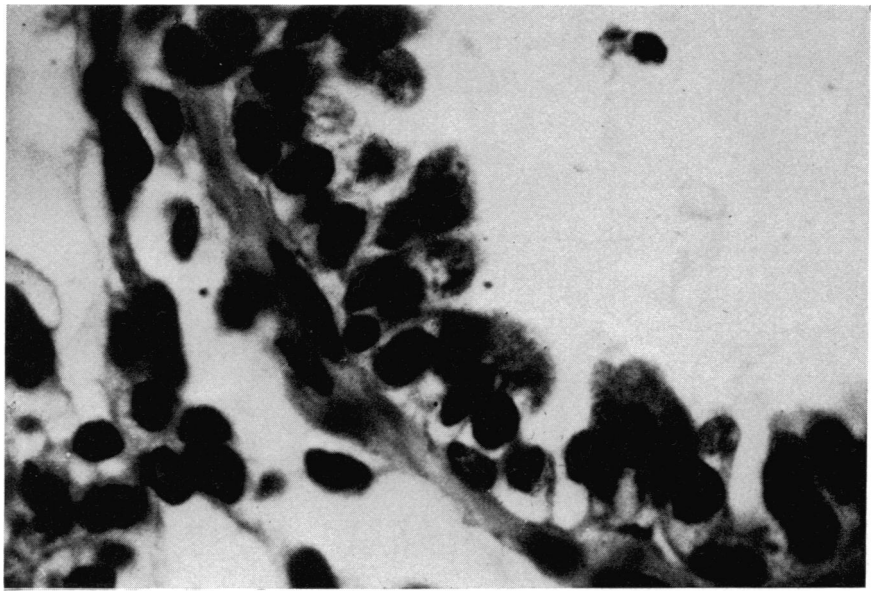

FIG. 6. Cross-section of bronchus from Sendai virus-infected cyclophosphamide-treated mouse, day 3. The bronchial basement membrane is thickened, but otherwise virtually intact. (PAS; magnification ×4025.)

reported increase in immunoglobulin-containing cells at day 3 in S mice (Blandford *et al.*, 1971). This was not present in SC mice at this time, but was detectable in some areas by day 6. In these areas the BBM was usually damaged as described above and the infected cells were desquamated. Pneumonic areas of the lungs of SC mice which died at days 7–9 showed the presence of fair numbers of immunoglobulin-secreting cells. Pneumonic changes were rare in S mice so that detailed comparison with SC mice were not possible at this level of the lung. Ig cells were rarely seen in the lungs of C mice and their total numbers in SC mice were much less than in S mice.

RENAL STUDIES

In the first mouse experiment dramatic differences between S and SC animals were detected when the kidneys were stained for immunoglobulin. On days 3, 6 and 9, S mice showed extensive granular deposition of gammaglobulin in most glomeruli (Fig. 7). In contrast N and C mice and day 0 animals were completely negative (Fig. 8). SC mice showed no deposits at day 3, scant deposits at day 6 (Fig. 9) and negative staining again at day 9.

Examination of these sections for Sendai antigens were initially negative. After acid elution of renal sections at pH 2·8, however, scattered deposits of Sendai virus antigen were detected by immunofluorescent techniques in S mice at days 3, 6 and 9 and in SC mice at day 6.

Seven repeat experiments have since been performed in mice infected with Sendai virus and two experiments in mice infected with avirulent influenza A virus (Kunz strain) in an endeavour to investigate this phenomenon further. An immediate problem was

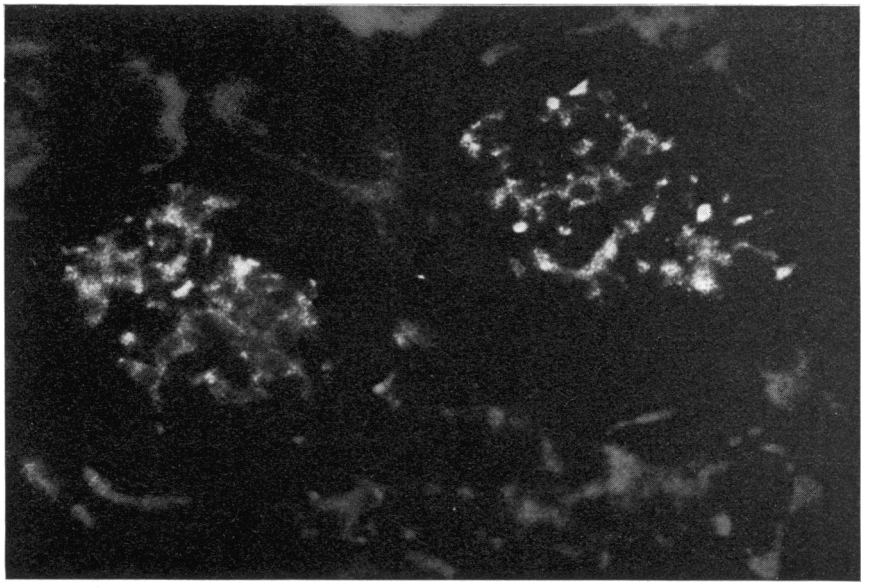

FIG. 7. Immunofluorescence staining of glomeruli of Sendai-infected mouse stained for mouse IgG. This appearance was seen on days 3, 6 and 9 and shows extensive granular deposition. (Magnification × 1610.)

FIG. 8. Immunofluorescence staining of glomeruli of control uninfected mouse stained for IgG, showing absence of staining. (Magnification × 1610.)

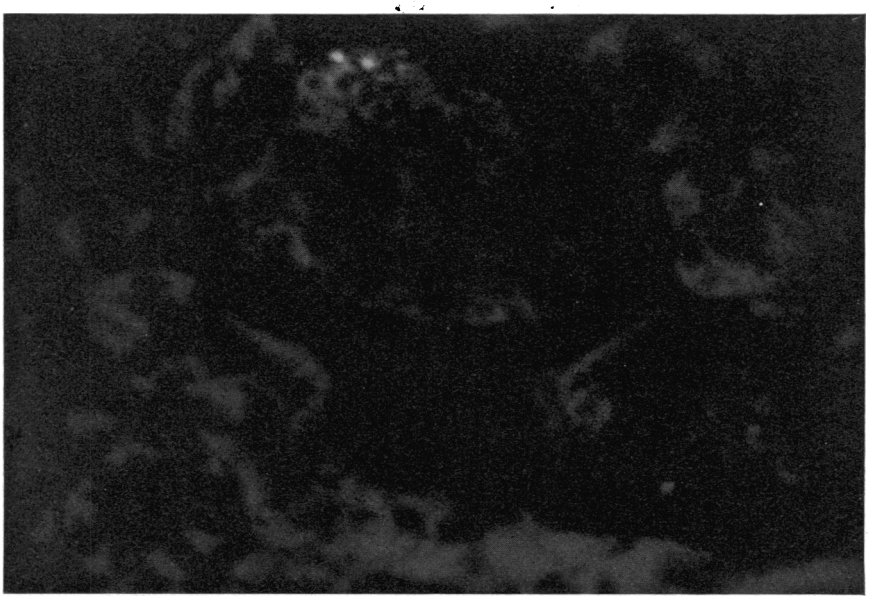

FIG. 9. Immunofluorescence staining of glomerulus of Sendai virus-infected, cyclophosphamide-treated mouse stained for IgG. The appearance at day 6, showing scant granular deposition in a localized area of glomerulus. (Magnification × 1610.)

encountered as all subsequent batches of mice including syngeneic strains of C3H, C57 Bl and the F1(C3H × C57 Bl) hybrid were found to have immunoglobulin deposits in their glomeruli before they were infected with virus. Ig deposition could not therefore be used as a parameter for renal involvement. Studies for viral antigen after elution of tissue sections, however, showed variable glomerular deposition in four of the seven Sendai studies and in one of the two Kunz virus studies. Viral antigens were not detected after day 12 in any experiment. These preliminary studies also showed that C3H mice developed more glomerular deposition with Sendai virus than C57Bl mice, the F1 hybrid gave an intermediate result and the positive findings may have been dose dependent. This suggests that at least transient immune complex deposition is a common occurrence after a trivial respiratory virus infection and that the immune response and genetic make up of the animal, as well as virus dose are important related factors. These studies are being extended and will be reported in detail elsewhere.

All the above results are summarized in Table 1. It can be seen that cyclophosphamide treatment resulted in failure to limit or eradicate virus, diminished and delayed the appearance of Ig-secreting cells and the production of local and serum antibody, reduced and delayed bronchial basement membrane damage, and significantly reduced the incidence of immunoglobulin and viral antigen deposits in the kidneys. Not shown is the appearance at day 6 and day 9, in SC mice, of severe pneumonia in lung parenchyma, associated with the presence of viral antigen, Ig-secreting cells and local antibody.

DISCUSSION

The previously reported effects of cyclophosphamide on the non-lethal Sendai virus infection of mice (Robinson *et al.*, 1969) have been confirmed in this study. In essence,

TABLE 1

SUMMARY OF THE ESSENTIAL FINDINGS OF THE BASIC EXPERIMENT

	Control animals	Sendai virus			Cyclophosphan Sendai virus	
		Days			Days	
		3	6	9	3	6
Lung virus*	−	+++	+	−	++++	+++
Peribronchial Ig cells*	±	++	++++	++++	±	++
Serum antibody†	−	−	1:64	1:128	−	−
Lung antibody*	−	++	+	−	−	+
Bronchial basement membrane damage	−	+++++	+++	+	±	+++
Glomerular Ig deposits*	−	++	+++	+++	−	+
Glomerular viral antigen*	−	±	++	+	−	±

−, ±, +, ++, etc. Indicate relative amounts.

* Detected by immunofluorescent techniques.

† Haemagglutination inhibition.

virus was not eradicated, serum antibody was not detected, the influx of mon cells into the lungs was diminished and the animals died at 7–9 days with a c pneumonia. This would indicate an important protective role for the missing a response in SC mice. However, the histological studies reported here indicate tl aspects of the local and even remote tissue damage could be attributable to tl specific immune response with its resultant amplification of non-specific inflar mechanisms. In particular, desquamation of infected cells, BBM damage, and complex deposition in glomeruli were only found when the specific immune resp partially recovered in SC mice.

BBM changes similar to those reported here have been commented on brie non-lethal influenza infection in man (Walsh, Dietlein, Low, Burch and Mc 1961). Reports of pathological studies of animals have generally made no comme BBM changes, but examination of photomicrographs in the literature all show the reported here, on or after the 3rd day. This has been so with influenza and parai infection in the mouse (Loosli, 1973; Appell, Kovatch, Reddecliff and Geron Newcastle disease virus in chickens (Burnstein and Bang, 1958), hamster parai infection (Blandford, to be published) and many other influenza infections o models, cited by Steele (1961).

Close examination of the apparent contradiction that the primary immune contributes to tissue damage leads to the conclusion that at least some local tissue must always result from the local immune responses in the microenvironment of a ing immunogenic stimulus, particularly if the antigens involved are replicat kinetics of the two processes result in a moment in time when local immune c must be formed. In the case of the lung, such complexes, entering the circulatic easily reach the kidney, as there is no intervening reticuloendothelial barrier, a be regularly deposited in glomeruli. In addition, cytolytic antibody to cell-as or BBM-associated viral antigens could arise at any time and could also ca damage. Delay or suppression of these factors would naturally result in only the c effects of the virus being present.

The ultimate demise of the SC mice with massive pneumonia could be attributable to the transfer of the immune reactants from bronchi to alveoli. In fact at days 6 and 9 in SC mice, viral antigens were extensively present in the alveoli together with evidence for systemic (WBCC rise) and local (Ig cells and antibody) escape from the immunosuppressive effects of cyclophosphamide. This fact that the virus and immune response finally meet at the respiratory surface rather than in the upper air passages could certainly compromise gas exchange and result in anoxia and death. Support for this contention can be found in the studies of cyclophosphamide-treated influenza A (PR8) infected mice (Singer, Noguchi and Kirchstein, 1972) and cyclophosphamide-treated influenza A (Kunz) infected mice (Heath, personal communication). In both of these studies of lethal infections, if the foregoing analysis is correct, death should be delayed by cyclophosphamide. This was in fact the case in both reports. In studies of the pathogenesis of virulent and avirulent variants of an influenza A (Kunz) virus infection in mice, the lethal outcome of infection with the virulent form of the virus could also possibly be attributed to the ability of this virus to reach and replicate in alveoli, resulting in a local immune response in this location and death with massive areas of lung consolidation (Raut, Hurd, Cureton, Blandford and Heath, 1975). This interpretation suggests that the immune response of the lung to virus infection could have differing effects on the host if the infection occurs at the respiratory surface (alveoli) rather than in the air passages (bronchi). The immune responses in these locations are probably qualitatively different. The bronchi constitute an external secretory surface with typical secretory humoral antibody responses (Blandford, 1970; Blandford and Heath, 1974). The responses in the alveoli may represent a qualitatively systemic, yet localized, humoral response. The cells in peribronchial tissue have been shown to have a ratio of IgA:IgG of 4:1 changing to 1:1 immediately after infection (Blandford and Heath, 1974), whereas the distribution in alveoli has recently been shown to be 1:9 (Blandford, unpublished data), much more like central lymphoid tissue. The implication of this could be that non-complement-fixing secretory IgA antibody in the air passages could modulate the potential damage of complement-fixing IgG antibody. In the alveoli no equivalent modulating influences appear to be present.

Against the suggestion that antibody is mainly responsible for the lung damage is the report that intranasally administered, heterologous anti-Sendai serum, which would presumably be mainly IgG antibody, failed to cause excessive pathogenic effects in Sendai-infected mice (Mims and Murphy, 1973). As there would presumably be a limit to the quantity of viral antigens produced in such an infection and the mice were not immunosuppressed, there might not have been a sufficient additional quantity of immune reactants to cause extra tissue damage. Reconstituting immunosuppressed mice with intranasally administered antibody would perhaps be a more informative experiment and such a study is currently in progress in my laboratory.

Although cyclophosphamide may have a direct effect on the chemical composition of basement membranes (McIntosh, Kihara, Kaufman and Kulvinskas, 1972), it is likely that its effects in these studies are the result of suppression of the primary humoral antibody response (Hoffsten and Dixon, 1974) and resultant delay in amplification of nonspecific inflammatory responses.

In the dose used in this report, cyclophosphamide had been shown to spare thymus-dependent lymphoid tissue (Turk and Poulter, 1972) suggesting that cell-mediated immunity is probably not affected and therefore neither responsible for virus eradication

nor tissue damage. It has also been shown that anti-lymphocyte and a potent anti-mouse thymocyte antiserum had no effect on the susceptibility of mice to influenza or parainfluenza infection (Hirsch and Murphy, 1968; Mims and Murphy, 1973), again suggesting that cell-mediated immunity plays no part in these infections. Nevertheless, systemic delayed hypersensitivity certainly follows parainfluenza and influenza infections of guinea-pigs (Weatherbee, 1973) and a form of local cell-mediated immunity follows local administration of an inactivated influenza vaccine (Waldman, Spender and Johnson, 1972). It is uncertain if the local cell-mediated immunity cell population studied by Waldman *et al.* (1972) was derived from the bronchi or washed out from lung alveoli. This distinction may be of some considerable importance in analysing the mucosal and parenchymal lung defense mechanisms. In any case, the possible role of local cell-mediated immunity in lung infections requires further careful study.

The occurrence of local lung damage at least partly due to the intact specific antibody response has clear-cut implications for vaccine manufacture and use. The analysis of the *in vivo* biological properties of induced and augmented antibody responses is urgently needed.

Extended studies of the apparent immunological glomerulopathy which followed trivial respiratory virus infection of mice are in progress and will be reported elsewhere. The presence of Ig deposits in some batches of normal mice before infection, has been previously reported (Markham *et al.*, 1973) and is presumed due to chronic murine leukemia virus infection (Pascal, Koss and Kassel, 1973; Porter, Porter and Cox, 1973). The possible importance of the renal findings presented here, both with respect to interpretation of renal biopsy material and the possible pathogenesis of many types of acute and even chronic renal diseases in man, cannot be over-emphasized.

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