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Experimental Infection of Rhesus Monkeys with Type D Retrovirus

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The naturally occurring immunodeficiency syndrome of macaque monkeys is an important animal model for the acquired immunodeficiency syndrome in humans. A new type D retrovirus, distinct from Mason-Pfizer monkey virus, has been isolated from affected animals at the New England Regional Primate Research Center. We now report the results of experimental infection of macaques with retrovirus D/New England after 13 months of study. Inoculated macaques developed lymphadenopathy without follicular hyperplasia, profound neutropenia, and a transient decrease in peripheral blood lymphocyte blastogenic responsiveness. Despite our varying the strain of virus, the manner in which the virus was grown, the size of the inoculum, and the age of the inoculated animals, infected macaques have not developed opportunistic infections or profound, prolonged loss of T cell function, key features of the macaque immunodeficiency syndrome. Therefore, experimental infection of naive macaques with D/New England has not reproduced the naturally occurring macaque immunodeficiency syndrome.

The immunodeficiency syndrome of macaque monkeys has many parallels to the acquired immunodeficiency syndrome in humans (7, 11). Animals with this syndrome develop profound, prolonged T lymphocyte dysfunction and eventually die of lymphomas or opportunistic infections by a spectrum of agents similar to those seen in human acquired immunodeficiency syndrome. We have shown that the macaque immunodeficiency syndrome can be transmitted to previously healthy macaque monkeys by using filtrates of lymphoma tissue from these animals (8, 10). Such studies implicate an infectious agent in this syndrome.

Our recent isolation of a new retrovirus from macaques with the immunodeficiency syndrome is therefore of extreme interest (4). This type D retrovirus is related to, but distinct from, Mason-Pfizer monkey virus, a retrovirus previously isolated from a rhesus monkey mammary tumor (3). We have cloned replicative intermediate (Hirt supernatant) DNA from this new type D retrovirus, derived detailed restriction endonuclease maps, and compared these with Mason-Pfizer monkey virus and squirrel monkey type D retrovirus maps. Although some restriction endonuclease sites are conserved, most sites are not (4).

The fact that this newly isolated virus is a retrovirus, a member of a family of viruses capable of inducing immunosuppression and malignancies (1), makes it a plausible candidate as the etiological agent in the macaque immunodeficiency syndrome. We have isolated this new type D retrovirus from 13 macaques with clinical evidence of disease, but isolation attempts from healthy macaques have repeatedly failed (4). We now report the results of inoculations of this newly isolated type D retrovirus into rhesus monkeys after 13 months of study.

Virus isolates. The type D retrovirus isolates used in these inoculation studies included two (D398 and D184) isolated from individual *Macaca cyclopis* with well documented, naturally occurring macaque immunodeficiency syndrome. These isolations were done by cocultivation of peripheral blood lymphocytes (PBL) from these animals with Raji cells, as previously described (4). Virus stocks frozen after less than 1 month in culture were used to prepare fresh virus stock for these inoculations. Another isolate (D225) was derived from a thymus explant culture from a *Macaca mulatta* with experimentally transmitted immunodeficiency disease. This animal developed the macaque immunodeficiency syndrome after receiving a filtrate of a lymphoma tissue homogenate from a macaque with an experimentally transmitted lymphoma. This isolate was never passaged in Raji cells.

Clinical and pathological studies. Macaques were examined clinically on a daily basis for the first month after inoculation and then on a weekly basis. Peripheral venous blood for hematological, immunological, and virological testing was drawn 1, 2, 4, and 8 weeks after inoculation. Such studies were done every 2 months thereafter.

The routine hematological studies reported here were carried out in the clinical laboratory at the New England Regional Primate Research Center. PBL were assayed for their proliferative response to pokeweed mitogen, concanavalin A, and *Candida* antigen as previously described (10). Mixed lymphocyte reactions with mitomycin C-treated *Saguinus oedipus* B cell lines as stimulator cells were carried out as previously described (10). Staining and analysis of PBL for cell surface antigens were carried out as previously described (12).

Lymph node biopsies were performed on all the macaques 4 weeks after virus inoculation. Hematoxylin-and-eosin-stained sections were prepared from 10% neutral buffered, Formalin-fixed tissue by standard histological techniques. Lymph node tissue was stained with lymphocyte subset-specific monoclonal antibodies as previously described (2).

Inoculation of macaques with type D retrovirus. Thirteen monkeys were inoculated with D/New England during a 13-month period. A number of parameters, including the particular virus isolate, the manner in which the virus was grown, the quantity of virus inoculated, and the age of the inoculated animal, were varied to optimize the likelihood of inducing disease in these monkeys (Table 1).

In spite of the many variables introduced into these studies, the clinical responses of the rhesus monkeys to the inoculation of the type D retrovirus have been remarkably consistent. All of the macaques developed a fever and axillary and inguinal lymphadenopathy 1 to 2 weeks after

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TABLE 1. Animals inoculated with type D retrovirus

Animal inoculated[a]	Age at inoculation (mo)	Virus isolate[b]	Cultivated in:	Quantity of virus[c]	Days post-inoculation
Mm 159-82	12	D398	Raji cells	10^4	390
Mm 214-82	12	D398	Raji cells	10^4	390
Mm 127-83	3	D398	Raji cells	10^4	360
Mm 106-83	3	D398	Raji cells	10^4	360
Mm 179-83	5	D184	Raji cells	$>10^9$	270
Mm 227-83	4	D184	Raji cells	$>10^9$	270
Mm 166-83	5	D184	Raji cells	$>10^9$	270
Cj 158-83	9	D225	Thymus explant culture	10^6	140
Cj 321-83	3	D225	Thymus explant culture	10^6	140
Mm 353-83	1	D225	Thymus explant culture	10^6	140
Mm 356-83	1	D225	Thymus explant culture	10^6	138[d]
Mm 27-84	1 day	D225	Thymus explant culture	10^6	140
Mm 28-84	1 day	D225	Thymus explant culture	10^6	102[d]

[a] Mm, *M. mulatta*; Cj, *C. jacchus*. Virus in 1 to 5 ml of medium was inoculated intravenously, subcutaneously, intraperitoneally, and intranasally into each animal.
[b] See text for details.
[c] Syncytia-forming units determined by serial dilution onto Raji cells; for inoculation of $>10^9$ U, virus was concentrated 500-fold.
[d] At time of death.

inoculation. This lymphadenopathy, although easily demonstrable, was not dramatic, and was no longer present 4 to 6 weeks after inoculation.

Virus isolations. Rhesus monkeys were selected for use in the study after the ability to isolate type D retrovirus from their peripheral blood was assessed and their antibody response to this virus was determined. Virus isolation was attempted by cocultivation of PBL with Raji cells, and antibody status was determined by an indirect immunofluorescence test. The animals used in the study showed no evidence of type D retrovirus infection before experimental inoculation. Cocultivations of all the macaque PBL yielded virus by 1 to 2 weeks after inoculation. Furthermore, type D retrovirus has been repeatedly isolated from these macaques as long as 9 months after inoculation. This type D retrovirus was frequently recovered from the cell-free plasma of infected macaques, suggesting that these animals remained viremic for a significant period.

Attempts to isolate other viruses from the macaques have only been made routinely on the first four rhesus monkeys listed in Table 1. Oral and anal swabs and PBL from these animals were cultured on human embryonic lung and Vero cell lines. These cultivations yielded only adenovirus isolates.

Studies done over a 6-week period after inoculation provided no evidence that *Callithrix jacchus* can be infected with this agent.

Hematological status of inoculated macaques. A consistent hematological picture was seen in the macaques after inoculation with the type D retrovirus. The data on the first seven animals listed in Table 1, those which have been followed for more than 270 days, indicate that all the monkeys developed a significant neutropenia. By 10 to 23 days after inoculation, these macaques had a mean total granulocyte count of 1,300 (range, 360 to 1,750). Moreover, this neutropenia persisted, with all the animals having total granulocyte counts of <2,000, and five of the seven animals occasionally having counts of <700 during the entire postinoculation period. The mean total granulocyte count of these animals before inoculation was 3,530.

Although these animals also developed an anemia, it was transient. By 7 to 30 days after inoculation, their mean hemoglobin concentration was 10.6 g/dl (range, 9.6 to 11.3). These values returned to normal in all the monkeys by 20 to 60 days after inoculation. None of the animals developed a persistent monocytosis or circulating mononuclear cells with prominent nucleoli and vacuolated cytoplasm (characteristic findings in the macaque immunodeficiency syndrome).

Of the two macaques inoculated at 1 month of age, one (353-83) developed a persistent neutropenia, but in the other animal, which died (356-83), the neutrophil count remained normal. Both macaques inoculated at 1 day old developed neutropenia; however, in the one which died (28-84), the neutrophil count was normal 10 days before death.

Immunological status of inoculated macaques. Although minor variations in PBL blastogenic responses to mitogenic and antigenic stimulation occurred when comparing one inoculated macaque with another, the responses of the PBL of the animals shown in Fig. 1 and 2 were characteristic of the pattern seen in these animals. By 7 days after inoculation, the proliferative responses of PBL from these animals were decreased compared with those of the controls. The proliferative responses of PBL from the macaque shown in Fig. 1 normalized by 1 month after inoculation and have remained normal since that time. This transient change was typical of the immunological abnormalities seen in these inoculated monkeys. The longer period of decreased PBL responsiveness shown by cells from another animal represented one of the most abnormal immunological profiles seen in these inoculated macaques (Fig. 2). However, in no case was the PBL responsiveness of these monkeys profoundly decreased, nor did it remain depressed for a prolonged period. The absolute number of circulating T4 (helper/inducer) and T8 (suppressor/cytotoxic) T cells in the animals did not change significantly throughout these studies.

Histological appearance of lymph nodes. Sheets of mature-appearing lymphocytes containing occasional cells with an immunoblastic or histiocytic appearance were seen on hematoxylin-and-eosin-stained sections of lymph nodes. There was no evidence of the marked follicular hyperplasia or lymphoid depletion characteristically seen at various stages of the macaque immunodeficiency syndrome. Follicles were noted when the sections were stained with monoclonal antibodies specific for lymphocyte subsets. The overwhelming number of paracortical lymphocytes were T4 bearing when assessed by this technique. T8-bearing cells predomi-

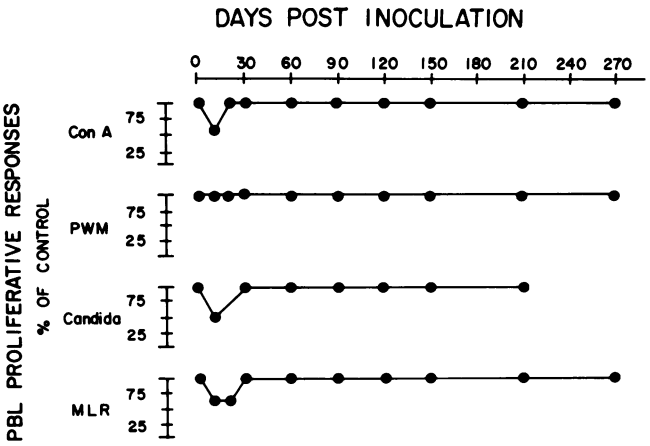

FIG. 1. PBL from a D/New England retrovirus-inoculated rhesus monkey, showing a transient decrease in blastogenic responsiveness. The proliferative responses of PBL from this animal to lectin and antigen stimulation are expressed as a percentage of the response shown by PBL from two age-matched control rhesus monkeys assayed simultaneously.

nate in the paracortex of lymph nodes early in the course of the macaque immunodeficiency syndrome (1a).

Necropsy findings. Two animals, both under the age of 4 weeks at the time of inoculation, died during this study. Both had a terminal diarrheal illness without associated gastrointestinal histopathological changes. Thymic atrophy was noted in both macaques, with an apparent secondary depletion of lymphocytes in the spleen and lymph nodes of one of them. One animal (28-84) had an aspiration pneumonia and inclusion bodies in hepatocytes comparable to those previously associated with paramyxovirus and not infrequently seen in immunologically normal monkeys (9).

Conclusions. The clinical picture seen in the rhesus monkeys inoculated with the retrovirus D/New England was remarkably consistent in spite of variations in the age of the animals, in the source of virus inoculum, and in the quantity of virus inoculated. The most unusual change seen in these inoculated animals was the profound neutropenia. Although neutropenia is a common transient phenomenon in a variety

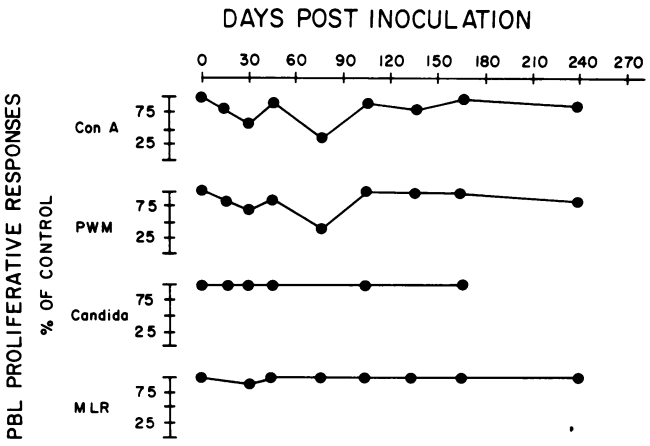

FIG. 2. PBL from D/New England retrovirus-inoculated rhesus monkey Mm 106-83, showing a more prolonged decrease in blastogenic responsiveness. The proliferative responses are expressed as described in the legend to Fig. 1.

of viral syndromes, its persistence in these macaques was quite striking. The other pathological changes caused by this agent in macaques immediately after inoculation, the morphological changes in the lymph nodes and the transient depression of blastogenic responsiveness of PBL, are characteristic of a variety of viral syndromes. Some of the findings described here were also noted in a previous study of newborn rhesus monkeys inoculated with Mason-Pfizer monkey virus (5).

Inoculated rhesus monkeys showed no evidence of profound, prolonged T lymphocyte dysfunction, no opportunistic infections, and no apparent lymphomas or lymphoproliferative abnormalities. The lymph nodes of these animals did not show the depleted appearance characteristic of the macaque immunodeficiency syndrome. These animals also did not develop the persistent monocytosis or circulating mononuclear cells with prominent nucleoli seen previously in this syndrome. The two inoculated rhesus monkeys which died showed none of the clinical or immunological abnormalities characteristically seen in the macaque immunodeficiency syndrome (11) and showed none of the characteristic pathological features of this syndrome at necropsy (9). Therefore, experimental infection of clinically healthy monkeys with the D/New England retrovirus has not reproduced the macaque immunodeficiency syndrome. This virus still may play a causative role in the macaque disease. Rapid in vitro attenuation of the virus, variations in the virulence of different virus strains, a critical age of the macaques at the time of infection, or additional factors could make it difficult to establish a relationship between the virus and the disease. Furthermore, these infected macaques may develop the macaque immunodeficiency syndrome or lymphomas after a latency period of more than 1 year. It is possible, however, that this type D retrovirus simply represents another of the many opportunistic agents present in this syndrome.

Marx et al. (13) and Gravell et al. (6) have reported that two of five and two of two inoculated macaques, respectively, died after inoculation with a type D retrovirus isolated from macaques at the California Primate Research Center in Davis. Whether these animals developed opportunistic infections and profound, prolonged loss of T-cell function, however, is not clear.

Continued investigation is required to determine the spectrum of pathological changes caused by type D retroviruses and what role they play, if any, in the macaque immunodeficiency syndrome.

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Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (D.H.E.W. publication no. [NIH] 78-23, revised 1978).

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