Immunodeficiency and Clonal Growth of Target Cells Induced by Helper-Free Defective Retrovirus

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The murine acquired immunodeficiency syndrome is induced by a defective retrovirus. To study the role of virus replication in this disease, helper-free stocks of defective Duplan virus were produced. These stocks were highly pathogenic in absence of detectable replicating murine leukemia viruses (MuLVs) other than xenotropic MuLV. They induced expansion of the infected cell population (over 1000-fold), and this cell expansion was oligoclonal in origin and, most likely, arose through cell division. These results suggest that this defective virus is oncogenic, inducing a primary neoplasia associated with an acquired immunodeficiency syndrome as a paraneoplastic syndrome. These data emphasize the need to determine whether virus replication is necessary for the progression of other immunodeficiency diseases, including acquired immunodeficiency syndrome, and whether these diseases also represent paraneoplastic syndromes.

ANIMAL MODELS ARE LIKELY TO BE important in gaining insight into the pathogenesis of human AIDS. A severe immunodeficiency disease induced by the Duplan strain (*1*) of MuLV has been described in mice (*2–6*). A defective retrovirus has been identified in diseased mice that, when pseudotyped with a nonpathogenic helper MuLV, induces typical disease (*7*). The role of virus replication in this disease was investigated by producing stocks of helper-free defective Duplan retrovirus with the Ψ2 packaging cell line (*8*). Cloned Du5HNeo DNA (*7*), containing the defective viral genome in the pSV2neo vector, was transfected in Ψ2 cells, and neomycin-resistant colonies were selected in the presence of G418 (200 μg/ml). One clone (out of 22) was selected as the best producer by its ability to secrete the highest levels of viral RNA hybridizing to a probe (D30) that is specific to the defective viral genome (*7*). The titers of these stocks were obtained by first infecting NIH 3T3 cells with serial dilutions of virus, followed by in situ hybridization of infected cells with ^{32}P-labeled D30 probe (*7*). Titers ranged from 1×10^3 to 1×10^4 infectious units per milliliter. The absence of replication-competent retrovirus in these stocks was assessed by infecting susceptible NIH 3T3 (Fv-1^n) and SIM.R (Fv-1^b) mouse fibroblasts. After 25 days of subculture, no replicating retrovirus was detected by the reverse transcriptase assay (*9*), confirming the helper-free nature of these stocks.

To study their pathogenic potential, the helper-free stocks of Du5H defective virus were inoculated intraperitoneally into young adult C57BL/6 mice. All inoculated mice developed clinically apparent disease within 12 to 15 weeks (Table 1). The disease was clinically similar to that induced by the defective virus pseudotyped with a replication-competent MuLV. The latency and the progression of the disease were also comparable whether a replicating helper was present or not. Splenomegaly or lymphadenopathy, or both, were apparent. Virtually all nodes were enlarged by 3 to 5 months after infection, some (submandibular and parathymic) reaching 1 g in weight. The functional assay for T lymphocyte response to the mitogen concanavalin A (Con A), performed as described (*7*), showed that in 11 of these diseased mice tested, the level of [^{3}H]thymidine incorporation, after Con A stimulation, was 10- to 30-fold (in one mouse, 3-fold) lower than in normal uninoculated mice, thus revealing that these mice had a profound suppression of their T lymphocyte function.

To confirm that no replication-competent helper virus was involved in this disease, spleen or lymph node cells (or both) from 12 diseased mice (3 months after inoculation) were cocultivated with susceptible mink cells and murine NIH 3T3 (Fv-1^n) and SIM.R (Fv-1^b) fibroblasts for 30 to 40 days, with frequent cell passages (*10*). No reverse transcriptase activity could be detected in the supernatants of NIH 3T3 and SIM.R fibroblasts cocultivated with cells from mice inoculated with helper-free stocks (Table 2), indicating the absence of replicating ecotropic, mink cell focus-forming virus, and dualtropic and polytropic MuLVs in these diseased mice. However, cocultivation of SIM.R cells with lymph node or spleen cells from mice inoculated with a pseudotype made of the defective virus rescued with a replication-competent B-tropic (G_6T_2) MuLV was reverse transcriptase–positive early, as expected. Mink cells cocultivated with lymphoid cells from normal mice or from mice inoculated with helper-free stocks showed positive reverse transcriptase activity in this assay (Table 2). This replicating virus could reinfect mink cells, but not NIH 3T3 or SIM.R cells, indicating that this virus belongs to the xenotropic class of MuLVs, previously found to be unable to replicate on mouse cells (*11*). No defective Du5H provirus expression could be detected in these mink cell cultures by the in situ hybridization procedure with D30 probe (*12*), suggesting the absence of Du5H (xenotropic) MuLV pseudotype in these mice.

The presence of the defective Du5H virus in these diseased mice was first assessed by extracting lymph node RNA and hybridizing with ^{32}P-labeled D30 probe (*7*). A major 4.2-kb RNA species, corresponding to the defective viral genome, was detected in all diseased tissues examined (Fig. 1A). A representative MuLV probe (BALB/c endogenous ecotropic MuLV), able to detect several copies of endogenous MuLV proviruses in the mouse genome, also detected the 4.2-

Table 1. Characteristics of the disease induced by helper-free stocks of Du5H defective MuLV.

Virus inoculated*	Number of mice diseased/ studied†	Spleen weight‡ (mg)	Lymph node weight§ (mg)	Latency‖ (weeks)
No virus	0/10	83–134	10–18	
Du5H (Ψ2)	30/30	130–1070	140–1350	12–15
Du5H (G_6T_2)	30/30	176–1010	410–870	12–15

*Helper-free stocks of defective virus Du5H (Ψ_2) (1×10^4 infectious units per milliliter) were inoculated intraperitoneally (1.5 ml) into young (30- to 40-day-old) C57BL/6 mice twice, 1 week apart. Defective virus rescued with a helper G_6T_2 MuLV, Du5H (G_6T_2), was also inoculated as control. †Diseased mice with lymphadenopathy or splenomegaly, or both at 16 weeks after inoculation. ‡As determined between 12 and 15 weeks after inoculation of five, ten, and seven mice in the no virus, Du5H (Ψ2), and Du5H (G_6T_2) groups, respectively. §The largest submandibular node of each mouse was weighed at 12 to 15 weeks after inoculation of five, ten, and seven mice in the no virus, Du5H (Ψ2), and Du5H (G_6T_2) groups, respectively. Normal mice had virtually no visible nodes. ‖Time at which the first clinical sign (lymphadenopathy) of the disease was observed.

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kb defective RNA species and an additional 5.2-kb RNA species, presumably an endogenous species. Also, a fainter band could be seen at 8.0 kb, the expected size of a full-length viral genome; this RNA probably originated from the xenotropic MuLV (Fig. 1B). Similar results were obtained with a representative and U3 LTR Moloney MuLV-specific probe (*13*). A MuLV-specific probe specific for the *env* ecotropic determinant (*14*) detected no RNA in mice inoculated with helper-free stocks (*13*), as expected, since the cocultivation assay was negative for a replicating ecotropic MuLV and since the defective viral genome had a deletion in this region homologous to the probe. However, in mice inoculated with a pseudotype made of the defective virus rescued with the replication-competent ecotropic G_6T_2 MuLV, the helper viral RNA could be easily detected with representative (Fig. 1B, lanes 10 and 11) or *env* ecotropic MuLV-specific (*13*) probe, as expected. Together, these results suggest that infected target cells in animals inoculated with helper-free stocks actively transcribed defective viral RNA in absence of helper RNA.

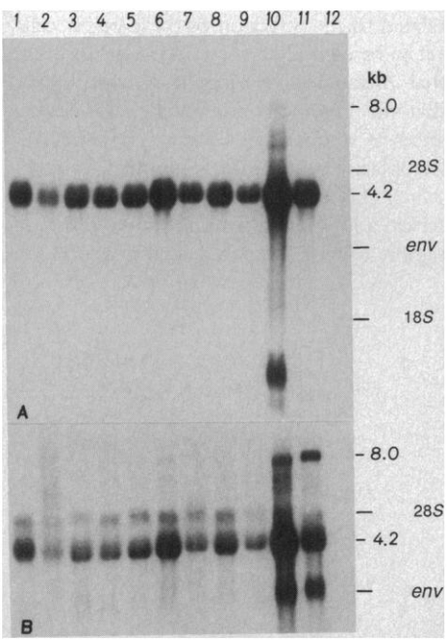

Fig. 1. Northern blot analysis of RNA from lymph nodes of diseased mice inoculated with helper-free stocks of defective virus. RNA was extracted as described (*23*) and separated by electrophoresis in 1% formaldehyde-agarose gels (*24*). After blotting on nitrocellulose membranes, the samples were hybridized with (**A**) a ^{32}P-labeled D30 probe (*7*), or with (**B**) a representative (comprising the complete 8.8-kbp viral genome of BALB/c endogenous ecotropic MuLV) probe. Total RNA (20 μg) was from lymph nodes of individual mice inoculated with helper-free stocks (lanes 1 to 9) or with defective virus rescued with helper ecotropic G_6T_2 MuLV (lanes 10 and 11), or it was from spleen of a normal mouse (lane 12). *Env* means spliced *env* mRNA. (A) and (B) represent the same filter that was first hybridized with the D30 probe, then washed and rehybridized with the representative probe.

The number of infected cells in enlarged nodes or spleen of diseased mice was determined by in situ hybridization performed on tissues or their dispersed cells with the ^{35}S-labeled D30 probe (*15*). This probe detected no positive cells in spleen cells from normal mice. In lymph node cells from mice inoculated with helper-free stocks of defective virus or with the defective virus rescued with replication-competent G_6T_2 MuLV, the D30 probe could detect about the same number of cells harboring high levels of defective viral RNA. The percentage of infected cells varied from 1.5 to 30% (most around 5%) in eight mice examined in four independent experiments. We calculated that each diseased mouse harbors about 1.5×10^9 cells within its enlarged diseased tissues, assuming that 0.1 g of lymph node represents 7×10^7 to 8×10^7 cells (as counted), and that a diseased mouse, 4 months after inoculation, harbors about 2 g of enlarged lymph nodes and spleen, as estimated by weighing organs. If 5% of these cells are infected with the defective virus, as detected by in situ hybridization, then our results indicate that a minimum of 7×10^7 cells harbor the defective viral genome. Since a total of only 3×10^4 infectious units of the defective virus were initially inoculated into each mouse in two injections, the maximum number of target cells initially infected was 3×10^4. Therefore, our data indicate that there are at least 1000-fold more infected cells in diseased mice than in newly inoculated mice.

Defective proviral DNA was searched in genomic DNA extracted from enlarged lymph nodes of mice inoculated with helper-free stocks with D30 or D34 probe derived from the *gag* p12 and *env* 3′ end region of Du5H, respectively (*7*). Although both of these probes also hybridized with endogenous viral sequences, newly acquired 4.2-kbp Pst I defective viral DNA fragment could be detected in each lymph node DNA with the D34 probe (Fig. 2A). From the intensity of the signals and a comparative experiment (*13*), we calculated that about 5 to 30% of the cells within these organs were infected, in concordance with the in situ hybridization data, and assuming one proviral copy per cell. The intensity of the signals varied but was comparable for DNA extracted from organs of mice inoculated with a replication-competent or a helper-free virus, again indicating that the presence of replicating viruses did not significantly increase the number of cells infected with the pathogenic defective virus.

To determine whether the cells infected with the defective virus constitute an oligoclonal or a polyclonal cell population, we looked for defective proviral DNA-cell junction fragments in genomic DNA of enlarged organs by restriction endonuclease analysis with the D30 or D34 probe (*16*). We could detect discrete newly acquired fragments of a distinct length in some individual lymph nodes of some mice inoculated with replicating (Fig. 2B), or helper-free (Fig. 2C) viruses, indicating clonality or oligoclonality of the infected target cells. However, by screening two lymph nodes of each mouse, such newly acquired fragments could not be detected in two of the four tested mice

Table 2. Detection of infectious retrovirus from diseased mice inoculated with helper-free stocks of Du5H defective MuLV. The passage level of the cocultivated cells was 2 to 5 by the end of the experiment, except for the LN cells used with mink as the susceptible indicator cells, which were at passage level 2 to 3. LN, lymph node; S, spleen.

Virus inoculated	Number of mice studied	Origin of cells cocultivated*	Susceptible indicator cells	Reverse transcriptase activity† (cpm)
No virus	3	S	SIM.R	<4,000
	1	S	NIH 3T3	<4,000
	1	S	Mink	129,552
Du5H (Ψ2)	12	S	SIM.R	<4,000
	8	S	NIH 3T3	<4,000
	6	S	Mink	250,442
	11	LN	SIM.R	<4,000
	5	LN	NIH 3T3	<4,000
	5	LN	Mink	65,496
Du5H (G_6T_2)	1	S	SIM.R	838,950
	1	S	NIH 3T3	<4,000
	1	LN	Mink	11,746

*Cocultivation assay was done essentially as described (*10*). †The reverse transcriptase activity was performed as described (*25*), except that ^{32}P-deoxythymidine triphosphate was used. Background of uninfected cells varied from 2000 to 4000 cpm. The number of counts per minute represents the highest number obtained.

inoculated with a replicating virus and in three of the ten tested mice inoculated with helper-free stocks, most likely because the high number of endogenous viral fragments hybridizing with these probes prevented detection in all inoculated mice. Also, not all infiltrated organs of the same mouse were found to harbor newly acquired fragments, suggesting that either these organs were infiltrated by a lower number of infected target cells, by a different clonal population, or by a population not yet clonal, making detection difficult. We documented the presence of a distinct proviral integration pattern in different nodes of the same mouse in eight animals (for example, mice 1-2, 2-0, 2-1, and 2-9, Fig. 2, B and C). However, the same pattern of newly acquired fragments could be detected in two distinct lymph nodes of one mouse (mouse 2-22) (Fig. 2C, lanes 6 to 8). In some lymph nodes, the same newly acquired viral fragment could be detected with both the D30 and D34 probes (Fig. 2C, lanes 13 to 16), strongly suggesting that the detected fragments were indeed derived from the defective Du5H provirus. Together, these results suggest that the infected target cells emerge as oligoclonal populations in the diseased mice.

The present work shows that mice can develop a retrovirus-induced immunodeficiency syndrome in the absence of a replicating virus. Only three diseases have previously been shown to develop after inoculation of helper-free stocks of defective retroviruses: erythroleukemia with the spleen focus-forming virus (*17*), pre-B cell lymphoma in BALB/c mice inoculated intraperitoneally with the Abelson virus (*18*), and T cell lymphoma with the Abelson virus inoculated intrathymically (*19*).

The target cells infected with the defective virus are not numerous and represent a proportion of the infiltrated diseased organs [which themselves represent a heterogenous population of proliferating polyclonal B, T, non-B, and non-T cells (*2, 5, 20*)]. The number of infected target cells was not very different in diseased mice inoculated with the defective virus pseudotyped with a replication-competent helper or with helper-free stocks. This also suggests the limited role of the helper virus in generating target cells infected with the defective virus and in the progression of the disease. This result also suggests that there may be few target cells in the body of the animal and that the final infected cell population may emerge after a long latent period after infection, as a consequence of selection. In such cases, the presence of an actively replicating virus might not have much of an effect on the final number of infected cells. However, in diseased mice, the number of infected cells was found to be at least 1000-fold higher than in newly inoculated mice. This result strongly suggests that the expansion of this cell population occurs from cell division and not from reinfection, because these mice were found to harbor no virus replicating on mouse cells. This conclusion is based on the assumption that the xenotropic MuLV, which is known to be unable to replicate on mouse cells (*11*), cannot reinfect these specific target cells either.

Our data indicate that this expanded cell population is clonal or oligoclonal and therefore emerged from the outgrowth of a single or a few infected cells in each node. These could have been transformed or have gained a growth advantage after infection by this defective retrovirus. Surprisingly, each infected cell clone in each organ often seems to emerge independently and does not appear to migrate elsewhere since, except for mouse 2-22, no node of the same diseased mouse was found to have the same pattern of newly acquired proviruses. In mouse 2-22, however, the same pattern of newly acquired proviruses was found in two distinct lymph nodes. If we assume, as it is likely, that the pathogenesis of the disease is related to the infection of these target cells, yet to be identified, then our results indicate that this defective virus is oncogenic and that this murine immunodeficiency disease arises as a consequence of a neoplasia (paraneoplastic syndrome). The mechanism by which this defective virus, which has conserved a long open reading frame in the *gag* region (7) and which encodes a p60 *gag*

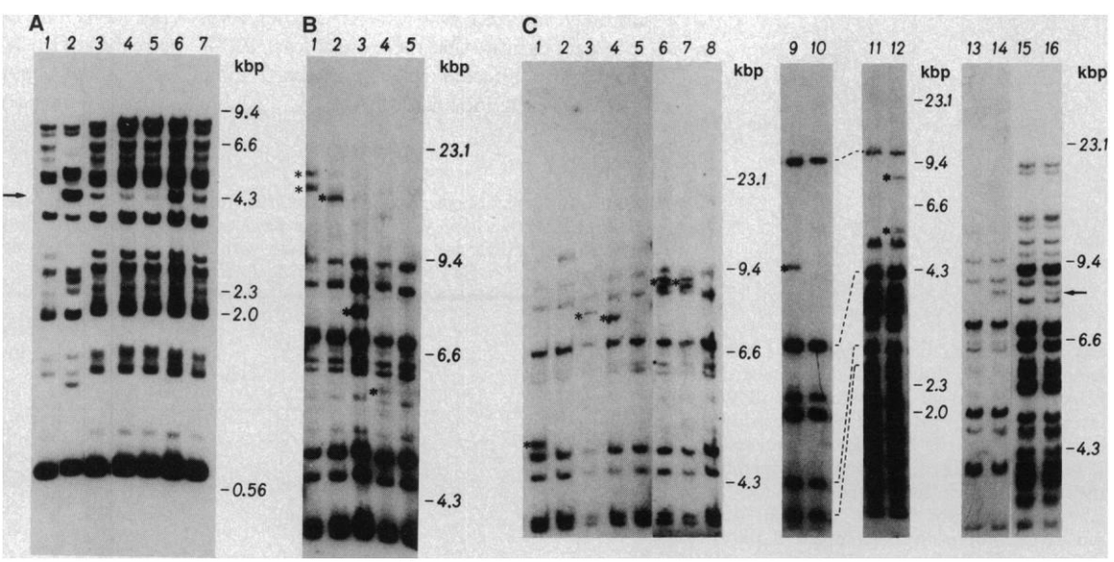

Fig. 2. Southern blot analysis of DNA from lymph nodes of diseased mice inoculated with helper-free stocks of defective virus or with replication-competent pseudotypes. (**A**) Cellular DNAs (20 μg) were digested by Pst I, and the fragments were separated on 1% agarose gels, transferred on nitrocellulose membranes, and hybridized with a ^{32}P-labeled D34 probe (nucleotides 3902 to 4184 of the Du5H sequence (7). DNA was from normal C57BL/6 mouse (lane 1), from G_6T_2 ecotropic MuLV-infected SIM.R fibroblasts harboring the defective Du5H viral genome (lane 2), from lymph nodes of three different mice inoculated with helper-free stocks of defective virus (lanes 3 to 5), or with defective virus rescued with G_6T_2 MuLV (lanes 6 and 7). ^{32}P-Labeled Hind III–digested λ DNA was used as a marker. The arrow shows the defective viral genomes. (**B**) Cellular DNAs from diseased mouse 1-2 inoculated with defective virus rescued with helper G_6T_2 MuLV were digested by Sac I and hybridized with the ^{32}P-labeled D30 probe. DNAs were from four different lymph nodes (lanes 1 to 4) and from the liver (lane 5). (**C**) Cellular DNAs from four mice (mouse 2-0, 2-1, 2-9, and 2-22), inoculated with helper-free stocks of defective virus, were digested by Sac I and hybridized with ^{32}P-labeled D30 probe (lanes 1 to 8 and lanes 13 and 14) or with ^{32}P-labeled D34 probe (lanes 15 and 16) or digested with Taq I and hybridized with ^{32}P-labeled D34 probe (lanes 9 to 12). DNAs were extracted from four different lymph nodes (lanes 1 to 4) and from the liver (lane 5) of mouse 2-1; from two lymph nodes (lanes 6 and 7) and from the kidney (lane 8) of mouse 2-22; from two lymph nodes of mouse 2-0 (lanes 9 and 10) and of mouse 2-9 (lanes 11 and 12). Sac I–digested DNAs from two lymph nodes of mouse 2-1 were first hybridized with the D30 probe (lanes 13 and 14); the same filter was washed and rehybridized with the D34 probe (lanes 15 and 16). The arrow shows the fragment hybridizing with both probes. For (B) and (C), an asterisk at the left side of a band indicates a newly acquired fragment.

precursor (*13*) modified in its p12 region (7), transforms cells is unknown. Since the final target cell population is clonal, the expression of the defective viral genome does not seem sufficient by itself to transform these cells and, most likely, another genetic event is required for their full transformation. Therefore, the initial causal disease appears to be a benign (hyperplasia or dysplasia) or a malignant neoplasia leading to a secondary immunodeficiency state. The infected target cells could be induced to secrete one or more factors detrimental to the immune system, directly or indirectly, or they could stop secreting factors essential for the normal function of the immune system. Alternatively, their interaction with other cells of the immune system could trigger the immunodeficiency.

AIDS patients carry few infected cells and show little virus replication (*21, 22*), a status that appears similar to the one seen in these mice infected with helper-free stocks of defective virus. Our results emphasize the need to determine whether virus replication is necessary for the progression of AIDS and whether this human syndrome is also the consequence of a neoplasia. If the primary defect in AIDS is a neoplasia, as it appears to be in this murine model, cofactors would be expected to play an important role in the initiation of the disease. One of the practical implications would be the immediate availability of several anticancer drugs to try to prevent the development of AIDS and to treat it.

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Cyclosporin A Specifically Inhibits Function of Nuclear Proteins Involved in T Cell Activation

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One action of cyclosporin A thought to be central to many of its immunosuppressive effects is its ability to inhibit the early events of T lymphocyte activation such as lymphokine gene transcription in response to signals initiated at the antigen receptor. Cyclosporin A was found to specifically inhibit the appearance of DNA binding activity of NF-AT, AP-3, and to a lesser extent NF-κB, nuclear proteins that appear to be important in the transcriptional activation of the genes for interleukin-2 and its receptor, as well as several other lymphokines. In addition, cyclosporin A abolished the ability of the NF-AT binding site to activate a linked promoter in transfected mitogen-stimulated T lymphocytes and in lymphocytes from transgenic mice. These results indicate that cyclosporin A either directly inhibits the function of nuclear proteins critical to T lymphocyte activation or inhibits the action of a more proximal member of the signal transmission cascade leading from the antigen receptor to the nucleus.

CYCLOSPORIN A (CsA) IS A CYCLIC undecapeptide with highly specific immunosuppressive effects that have been useful in treating rejection of allogenic transplants. Although the mechanism of action of this drug is unknown, it appears to act early to inhibit activation of T lymphocytes by antigen (*1*). These early effects on T cells give rise to various secondary effects on B cells, macrophages, and other cells dependent on the products of activated T cells, although CsA probably also has direct effects on cells other than T lymphocytes. Cyclosporin A does not inhibit the immediate membrane events associated with triggering the antigen receptor such as inositol 1,4,5-trisphosphate (IP_3) generation or calcium mobilization (*1, 2*). However, the transcriptional activation of lymphokine genes such as interleukin-2 (IL-2), IL-4, and gamma interferon (IFN-γ) are blocked (*3*). These results suggest that CsA interferes with the function of molecules that transmit signals between membrane events such as IP_3 generation and the activation of genes in the nucleus.

Several nuclear proteins have been implicated in the regulation of the early T cell activation genes (Fig. 1A). Activator protein 1 (AP-1), NF-κB, Oct-1, and AP-3 bind to sequences within the IL-2 or IL-2 receptor promoters essential for their transcriptional activation (*4–8*). A protein called "nuclear factor of activated T cells" (NF-AT) binds to sequences within the IL-2 enhancer necessary for both inducibility and T cell–specific expression. NF-AT appears to be the product of an earlier gene in the T cell activation pathway and probably accounts for the requirement for protein synthesis for IL-2 gene activation (*5*).

We initially used the human Jurkat T cell line to determine whether CsA alters the binding or function of these nuclear proteins because Jurkat cells mimic many of the early events of T cell activation (*9*). Both IL-2 and IL-2 receptor genes are activated by triggering the antigen receptor of Jurkat cells and, as with resting peripheral T cells, the activation of the IL-2, IL-4, and IFN-γ genes is inhibited by CsA (*3, 10, 11*), whereas the IL-2 receptor gene is relatively unaffected (*12*).

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