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CHARACTERIZATION OF HUMAN T CELL CLONES SPECIFIC FOR CORONAVIRUS 229E

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ABSTRACT

Coronaviruses (CV) are pleomorphic enveloped RNA viruses that are ubiquitous in nature, causing a variety of diseases in both man and domestic animals. In man, CV are generally associated with upper respiratory tract infections. The two prototype strains that are the best studied human CV isolates and which are thought to be responsible for most of the respiratory infections caused by CV are called 229E and OC43. Humoral responses consisting of neutralizing antibodies to CV are present in most individuals by six years of age. Although the cellular immune response to CV in man has not been characterized at all, it is known that the spike (S) and nucleocapsid (N) proteins elicit the major cell mediated immune responses in the mouse.

This report describes the production and characterization of eleven independently isolated T cell clones that are specific for the human CV(HCV) 229E. The T cell clones are $CD4^+$ and presumably recognize a processed viral peptide presented by class II molecules on the surface of antigen presenting cells. Of six 229E-specific T cell clones tested against purified viral proteins, three recognize the 180 kD spike glycoprotein while the other three recognize the 55 kD nucleocapsid phosphoprotein. Analysis of the human T cell mediated response to HCV will provide information regarding which viral proteins elicit the immunodominant response, what the fine specificity of these T cell clones are (immuno-dominant peptides), and what the T cell receptor (TCR) and cytokine usage is of these virus specific clones.

Corona- and Related Viruses, Edited by P. J. Talbot and G. A. Levy
Plenum Press, New York, 1995

INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease of the human central nervous system (CNS). The etiology of MS is unknown, although analysis of animal models of experimental allergic encephalomyelitis[1-3] as well as data from MS patients (reviewed in Ref. 4) indicate that both genetic and environmental factors contribute to this disease. Viruses are among the environmental factors that have been proposed as possible causative agents for MS. Although numerous viral agents have been implicated as candidates in the etiology of MS, no clear association between any particular virus and the disease has been confirmed[5].

Although CV infection in man is more frequently associated with upper respiratory tract infections, causing up to 35% of all cases of the common cold[6,7], there is evidence that CV are also involved in enteric infections[8,9] and childhood meningitis[10]. In the latter case, a coronavirus (Tettnang virus) was cultured from the cerebrospinal fluid of an 18 month old child with viral meningitis that followed an upper respiratory tract infection. The possible involvement of CV in MS was suggested initially by the observation of viral particles bearing the typical morphological features of CV in electron microscopic sections of brain taken from an MS patient[11], followed by a report describing the isolation of two separate CV from MS autopsy brain tissue[12,13]. Recently, our laboratory has identified murine-like coronavirus (MCV) RNA sequence and antigen in MS brain by in situ hybridization and immunohistochemical techniques[14]. Another group of investigators found human CV 229E sequences in MS brains by using the polymerase chain reaction[15], and also showed that the virus may be neurotropic due to its ability to infect a variety of human cell lines of CNS origin[16]. Following intracranial inoculation, CV can productively infect and disseminate in primate brains, resulting in encephalomyelitis and demyelination[17]. Similarly, MCV infections in rodents result in a panencephalitis accompanied by extensive demyelination[18,19]. Rodents infected with subacute levels of MCV JHM show evidence of chronic demyelination as a result of viral persistence within the CNS[20,21]. In mice both $CD4^+$ and $CD8^+$ T cells are required for CV clearance from the CNS and for prevention of encephalomyelitis[22,23]. Because the pathological changes in the CNS observed in rodents infected with JHM resemble those found in MS patients, this animal model has been used extensively to study both acute and chronic forms of demyelinating disease caused by viruses. We have chosen to characterize the human cell mediated response to 229E to determine whether there may be any relationship between CV and multiple sclerosis.

MATERIALS AND METHODS

Viruses and Cell Lines

The HCV strain 229E was grown on the human lung fibroblast cell line, WI-38. Both were obtained from American Type Culture Collection (Rockville, MD). The MCV strain JHM[24] was grown on the mouse tumor cell line DBT[25]. Both the virus and the cell line used for its propagation were originally obtained from Dr. Stephen Stohlman (University of Southern California, Los Angeles, CA). The cell lines were grown as monolayers in roller bottles using Dulbecco's modified Eagle medium (DMEM)(Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) in a humidified cell culture incubator at 37°C in an atmosphere of 5% CO_2. Virus infected cell monolayers were cultured at a lower temperature optimal for viral replication (34°C). Infected cell supernatants were harvested at 18 hr post-infection for JHM and 36 hr post-infection for 229E, with subsequent steps carried out at 4°C. Virus was purified from infected cell supernatants by precipitation with

0.5 M NaCl and 10% polyethylene glycol (mw 8,000; Sigma Chemical Co., St. Louis, MO), final concentration, followed by pelleting precipitated material at 10,000 x *g*. The virus was further purified by ultracentrifugation over two separate sucrose gradients, a 30%/50% discontinuous gradient centrifuged at 25,000 x *g* at 4°C for four hr followed by centrifugation of virus material through a 25%-55% continuous gradient at 25,000 x *g* for 18 hr. The purified virus band was removed from the gradient, pelleted by ultracentrifugation, and resuspended in sterile phosphate buffered saline (PBS) at a concentration of 1 mg/ml. Virus purity was determined by analyzing each preparation by polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL). The virus was completely inactivated by exposure to a combination of ultraviolet light (exposed for 15 min to a 15 watt 300 nm UV light source placed five cm from its surface), sonication and gamma irradiation (10,000 rad exposure from a cesium source IBL 437C cell irradiator; CIS-US, Inc., Bedford, MA) prior to being used in antigen presentation assays to prevent its replication in viable cells.

Autologous immortalized human B lines cell used as antigen presenting cells were obtained by transforming peripheral B cells using Epstein Barr virus (EBV). The EBV secreting transformed Marmoset lymphoblastoid cell line, B95-8 (originally obtained from American Type Culture Collection, Rockville, MD), was grown to high density, and the culture supernatant was centrifuged and filtered to remove cellular components. Peripheral blood lymphocytes (PBL) were obtained from heparinized blood by density gradient centrifugation over Histopaque-1077 solution (Sigma Chemical Co., St. Louis, MO). Approximately 1 x 10^7 PBL were incubated with 1 ml of EBV containing B95-8 supernatant for 1 hr at 37°C in a 10% CO_2 incubator. Cells were washed three times with RPMI 1640 medium (Gibco BRL, Grand Island, NY), resuspended to 2 x 10^6 cells per ml and plated into the first two rows of a flat bottomed 96 well plate at 200 µl per well. Subsequent rows contained serial two-fold dilutions of these cells. Ten µl of a 10 µg/ml stock of cyclosporin A (a generous gift of Sandoz Pharmaceuticals, East Hanover, NJ) was added to each well to inactivate the T cells in these cultures. The plate was fed weekly, with transformed B lymphoblastoid lines usually arising from three to five weeks after setting up the cultures. B cell lines were expanded as suspension cultures in Iscove's modified Dulbecco's medium (IMDM)(Gibco BRL, Grand Island, NY) supplemented with 5 x 10^{-5} M 2-mercaptoethanol, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Isolation of Purified 229E Nucleocapsid (N) and Spike (S) Proteins

Whole inactivated 229E virus was electrophoresed on 10% SDS-PAGE gels under reducing conditions. The gels were fixed and stained briefly with Coomassie brilliant blue dye to locate the N and S proteins, destained and then rehydrated in distilled water. The protein bands were excised from the gel, minced into 2-3 mm^2 pieces and placed into an electro-elution concentrator block (CBS Scientific, Del Mar, CA) in 0.05 M Tris acetate, 0.1% SDS buffer. Proteins were electrophoretically eluted from gel pieces overnight at 50 V. The buffer in the unit was changed to PBS and electrophoresis continued for two hr prior to harvesting the purified proteins.

Preparation of 229E Specific Human T Cell Clones

Transformed human B cells were used as antigen presenting cells to generate 229E specific T cell lines from autologous PBL. The B cells were incubated overnight with whole gradient purified 229E to allow for antigen (Ag) processing to occur, with 1 x 10^7 B cells being incubated with 250 µg 229E in 5 ml of fresh X-VIVO 15 medium (Biowhittaker, Walkersville, MD). Cultures containing freshly isolated autologous PBL were set up the

following day with irradiated (4000 rad) Ag pulsed B cells. PBL were isolated by gradient density centrifugation over Histopaque-1077. The buffy coat layer was transferred and the cells were washed three times in balanced salt solution. The PBL were mixed with the Ag-pulsed B cells resulting in final culture conditions of 5 x 10^5/ml PBL, 2 x 10^5/ml B cells and 5 μg 229E per well of a 24 well plate, with two plates being used. The cultures were incubated at 37°C in a humidified 10% CO_2 atmosphere for a total of ten days, being fed on day five with 1 ml of fresh medium. On day five (and on each day thereafter, up to day ten) eight wells were pooled and viable cells isolated by centrifugation over Histopaque-1077. The cells were cultured in fresh medium with 5% T-stim (contains IL-2, PHA and other T cell growth factors; Collaborative Biomedical Products, Bedford, MA) with 2 x 10^5/ml irradiated B cells as feeders. The cells were cloned in limiting dilution in 96 well plates using X-VIVO 15 medium with 8% T-stim and 2 x 10^4/well irradiated feeder B cells. Remaining bulk culture cells were frozen away for future use. The plates were fed 100 μl/well fresh medium with 20 U/ml recombinant human IL-2 (Collaborative Biomedical Products, Bedford, MA) on day five after cloning, and then fed every 5-6 days with fresh medium containing 8% T-stim. Wells that showed growth were expanded to 1 ml cultures using the same culture conditions and then tested in antigen proliferation assays five to seven days after expansion.

T Cell Proliferation Assay

T cells expanded to 24 well plates were tested in an Ag proliferation assay with Ag pulsed B cells irradiated just prior to setting up the assay. Generally, 5 x 10^3 to 1 x 10^5 viable T cells were cultured with 2 x 10^4 B cells with or without 1 μg/well 229E in flat bottomed 96 well plates in a final volume 200 μl. After two to three days, proliferation was measured during the final 18 hr of culture by the uptake of [^{3}H]-thymidine ([^{3}H]-TdR) and counted in a beta scintillation counter. Those cultures that responded well to whole 229E virus were reexamined after subcloning in a second proliferation assay with whole JHM virus (1 μg/well) and purified 229E N or S proteins (0.1-0.2 μg/well).

Subcloning of 229E-Specific T Cell Lines

T cell lines that showed a proliferative response towards 229E in the initial screening assay were expanded in fresh medium containing 20 U/ml human recombinant IL-2 for 3-5 days. The cultures were examined each day and the line was subcloned again in 96 well culture plates when it appeared that growth was optimal. Wells that showed positive growth were again expanded and tested for proliferation to whole 229E, with virus-specific subclones expanded for further study or to freeze down in reserve. Generally, 20 to 30 subclones were generated for each 229E-specific T cell line. The subclones were used in determining Ag dose response curves and to test Ag specific responses to the N and S proteins.

RESULTS AND DISCUSSION

This report describes the characterization of eleven independently isolated 229E-specific human T cell clones derived from two normal healthy donors. The 229E specific clones were obtained after culturing peripheral blood lymphocytes *in vitro* with virus pulsed APC. We assume that there are CV-specific T cells circulating in peripheral blood, considering the likelyhood of CV infections each year. A considerable amount of time was spent working out optimal conditions for the *in vitro* cultures and Ag presentation assays. Initially, primary *in vitro* cultures of PBL, APC and 229E were cloned from seven to fifteen days after onset.

In some cases after the primary stimulation with virus, cells were given a rest period by culturing in fresh medium with a small amount of growth factors (1% T-stim) for seven to ten days, followed by restimulation with 229E for an additional week. However, the majority of 229E-specific lines were derived from cells cloned from primary cultures that were five to seven days old (nine out of eleven). In our experience, reexposure of primary cultures to a second round of 229E stimulation did not increase the probability of generating 229E-specific lines.

The lines were selected on the basis of a high proliferative response to 229E virus pulsed APC relative to APC alone. Although the proliferative response was quantitated by the uptake of [^{3}H]-TdR, lines that responded well showed an obvious difference in the numbers of viable cells, which often appeared as clumps of cell blasts that were discernable by visual inspection alone. Typical responses of the eleven 229E-specific T clones are shown in Figure 1.

Stimulation indices (SI) for these T cell clones ranged from an SI of 5 (clone 2A.3) to 91 (clone 2C.9-3H.11). Six of the lines were isolated from one individual after screening a total of 558 clones resulting from two separate *in vitro* cultures, while the remaining five lines were isolated from another individual after screening a total of 410 clones resulting from a single *in vitro* culture. The frequency of clones isolated that responded well and maintained their response after subcloning corresponds to a 1.1% and 1.2% efficiency, respectively, although the actual numbers of clones selected after the initial screening was closer to 5%. Numerous lines that were initially selected based on a stimulation index of three to five were retested or subcloned, but, with one exception, the results of retesting the lines and subclones of marginal responders were uniformly negative. It is unclear whether

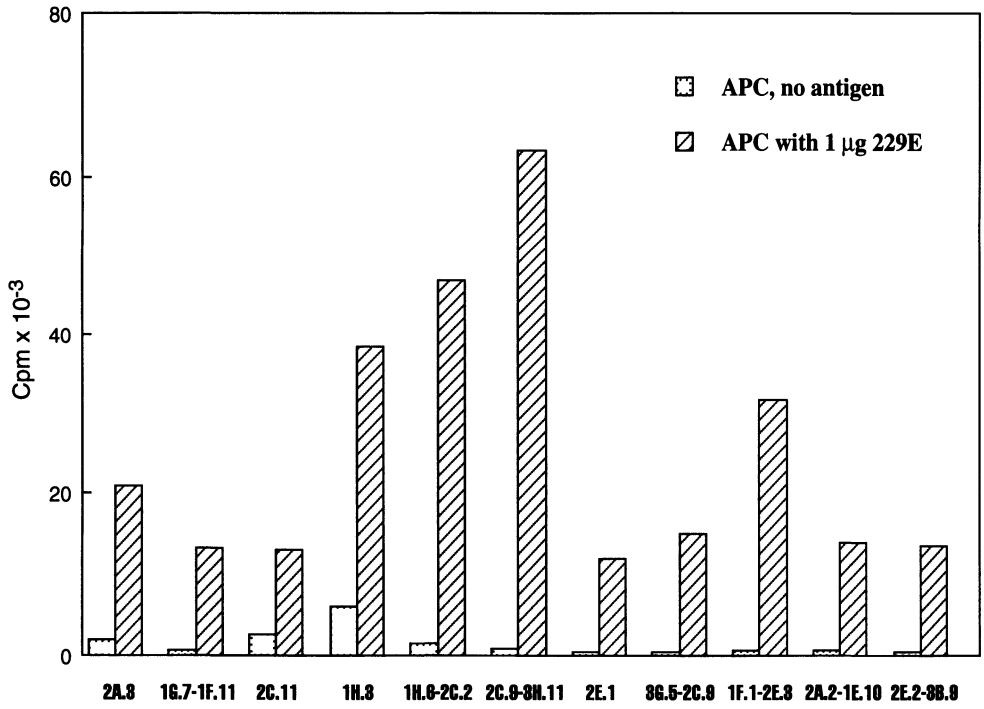

Figure 1. Response of 229E-specific human T cell clones towards 229E as detected in an Ag presentation assay. Approximately 2 x 10^3 to 1 x 10^5 T cells were cocultured with 2 x 10^4 autologous irradiated transformed B cells with or without 1µg of inactivated 229E After two to three days, proliferation was measured by the uptake of [^{3}H]-TdR during the final 18 hr period of culture and counted in a beta scintillation counter

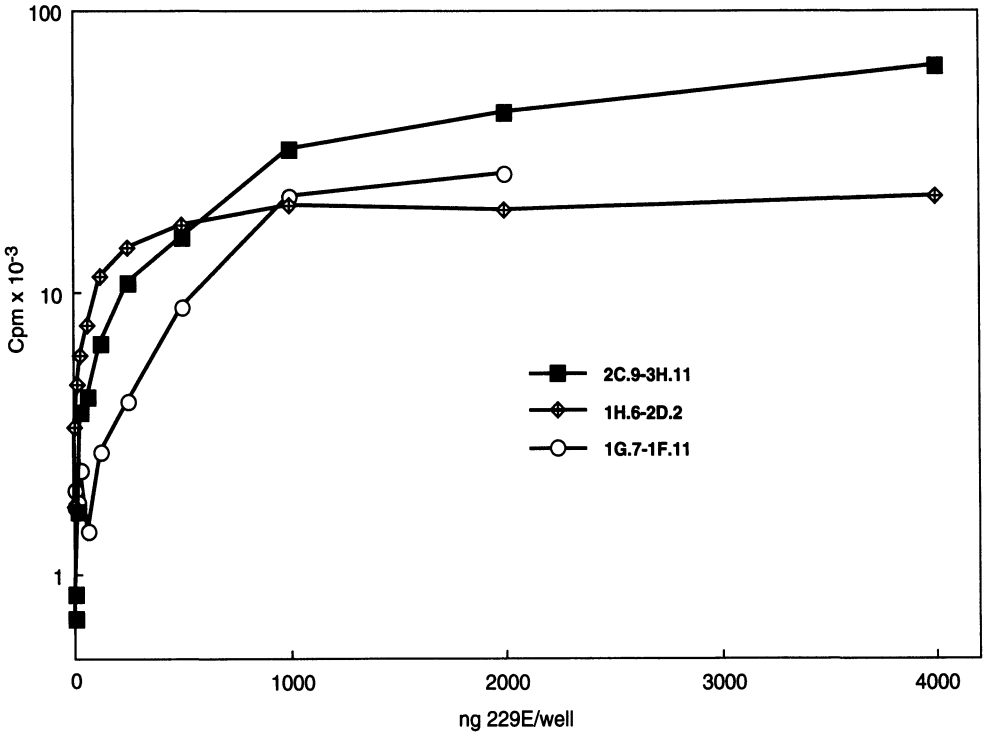

Figure 2. Dose response curve of 229E-specific T cell clones towards varying amounts of 229E. Autologous EBV transformed B cells lines were incubated overnight with serial two-fold dilutions of 229E ranging from 7 ng to 4000 ng per 100 ml. Approximately 2 x 10^4 Ag pulsed B cells per well were cocultured with T cells in triplicate. The number of viable T cells per well for each subclone was 2.0 x 10^4 for 1G.7-1F.11, 2.4 x 10^4 for 2C.9-3H.11, and 7.5 x 10^4 for 1H.6-2D.2. Proliferative responses were assessed by the uptake of [^{3}H]-TdR.

or not these weak responders consisted of multiple cell lines made up of a minor 229E-specific T cell population that was overgrown by a nonresponding line(s). All of the T cell clones characterized in this report are $CD4^+$ T cells, and therefore presumably recognize a processed viral peptide in the context of class II molecules on the APC.

To determine the limits of the response of 229E-specific T cell clones to antigen, a dose response curve was performed with three separate subclones by varying the concentration of 229E in the in the cultures from 7 ng to 4 μg per well, as illustrated in Figure 2. The half-maximal response for these subclones occurred with Ag amounts between 0.125 μg to 0.5 μg of 229E.

To determine the antigen specificity of the T cell clones, 229E N and S proteins were purified from SDS-PAGE gel slices by electroelution as shown in Figure 3.

We also wanted to determine if there was any crossreactivity of T cell epitopes on the MCV JHM, which is more closely related serologically to the HCV OC43 , but is antigenically unrelated to 229E[13,26]. The proliferative response of six representative T cell clones against whole virus and purified 229E N and S proteins is shown in Figure 4. As can be seen, three of the subclones tested responded to the N protein and three others responded to S. All of the T cell lines tested to date react with either N or S, indicating that these proteins probably elicit the immunodominant cell mediated responses in man, as had been previously reported for the mouse[23,27]. Currently, we are analyzing what peptides from the N and S proteins stimulate these 229E-specific T cell clones and what the TCR usage is on these clones.

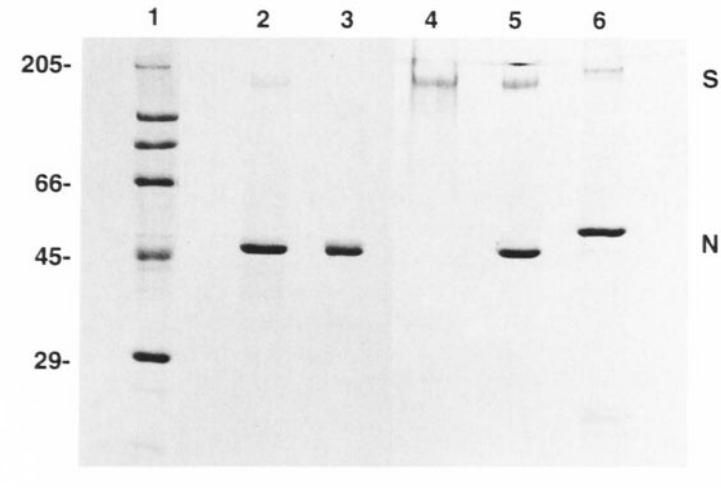

Figure 3. SDS-PAGE analysis of purified whole 229E and JHM coronaviruses and purified electroeluted 229E N and S proteins. Lane 1, molecular weight markers with numbers to the left indicating their relative mass in kDa; lanes 2 and 5, whole 229E; lane 3, 229E N protein; lane 4, 229E S protein; and lane 6, whole JHM. Approximately 1-2 μg protein was electrophoresed on a 10% polyacrylamide gel under reducing conditions. Lanes 1-3 were stained with Coomassie blue dye, while lanes 4-6 were developed using a silver stain to enhance the intensity of the S protein.

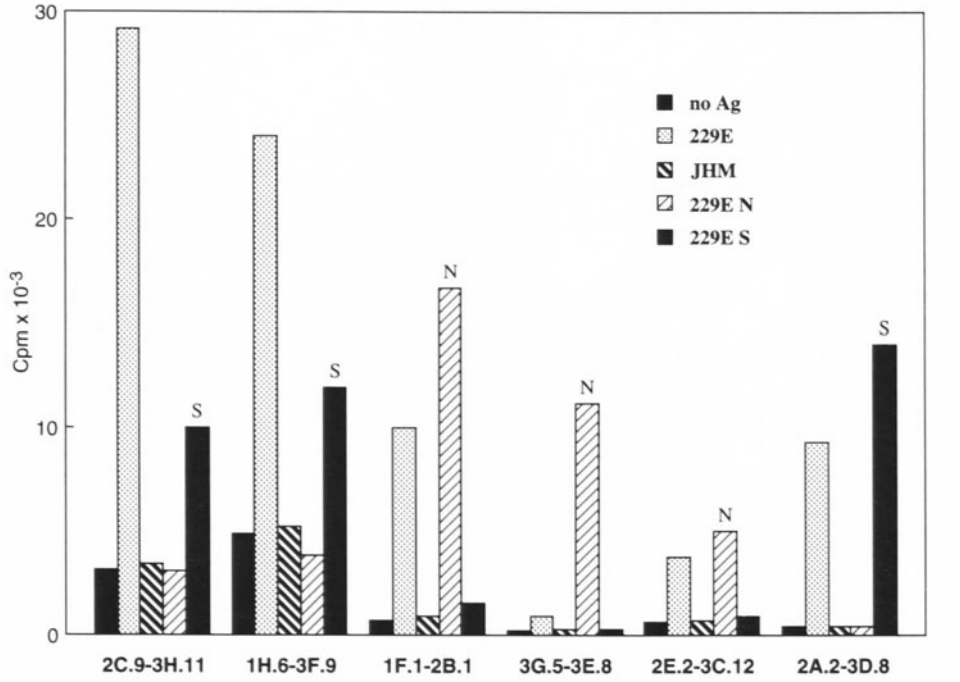

Figure 4. The proliferative response of six representative 229E-specific human T cell clones to a panel of antigens, including whole 229E and JHM virus (2 μg/well), and purified 229E N (0.2 μg/well) and S (0.1 μg/well) proteins. The reactivity patterns were confirmed by testing at least two individual subclones of each T cell line. The proliferation was determined by measuring the incorporation of [^{3}H]-TdR during the final 18 hr of culture.

In several autoimmune or chronic inflammatory diseases in man, such as rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, and Lyme disease, T cell mediated immunity is thought to play an important role in the pathogenesis of the disease. T cells isolated from the target organ involved have frequently been shown to express a restricted set of TCR molecules, supporting the notion that there is a preferential expansion of a subset of pathogenic T cells responding to an Ag stimulus. Knowing either what the offending antigens are (whether products of viruses or bacteria, or even self proteins) or the receptor usage of T cells involved in the disease process would allow for a specific target for pharmacologic intervention. In some instances, the putative target Ag and the TCR usage of T cells isolated from diseased tissue has been determined[28-30], while in other cases, an association between a particular TCR and an autoimmune disease exists, but the stimulating antigen is unknown[31,32]. The relationship between coronaviruses and MS remains an open question, but it certainly warrants further study.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Colorado Neurological Institute to J.S.S., support from Swedish Medical Center, and from the Nancy Davis Foundation. We are grateful to Dr. Judy van de Water (Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, CA) for helpful discussions in generating the human 229E-specific T cell lines, and to Joanne Streib (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) for assisting us in generating the EBV transformed human B cell lines.

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