

Identification of SIV/SMM Viral Proteins That Induce T Cell Response in Experimentally Infected Rhesus Macaques and Naturally Infected Sooty Mangabeys by the Cellular Western Blot Assay

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Initial studies have revealed subtle differences in the T cell proliferative response to whole SIV antigen in the peripheral blood mononuclear cells (PBMC) from sooty mangabeys and rhesus macaques. Preliminary findings utilizing the cellular Western blot assay are described.

Key words: T cell immunity • AIDS • CMI

INTRODUCTION

While considerable knowledge has been gained concerning the humoral response in HIV infection, relatively little is known concerning the cellular immune response. Cytotoxic T cells (CTLs) directed against HIV have been found in the peripheral blood of AIDS patients, but the protective nature of such a response is tempered by the identification of autoimmune lymphocytes as well [5,7,10]. In this regard, very little is known about the viral epitopes that are recognized in the cellular immune response. In an effort to delineate protective from pathogenic immunity, our laboratory has been examining the

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primate model for AIDS infection. By conventional virologic and serologic techniques, it has been determined that > 75% of the adult sooty mangabeys at the Yerkes Regional Primate Research Center (YRPRC) are naturally infected with SIV/SMM, without any apparent pathology or clinical sequela [4]. However, when rhesus macaques are experimentally infected with SIV/SMM isolated from mangabeys, they develop an AIDS-like disease which usually results in immunosuppressive disease and death [6]. Thus far, our laboratory has been able to document marked differences in a) the phenotype of PBMC [2], b) the phenotype and function of the cells mediating immunosurveillance [8], and c) the processing requirements and the magnitude of the antigen-specific cell-mediated proliferative response between these two species [3].

Our laboratory has developed an assay in which the proliferative response of nylon wool purified T cells to whole SIV/SMM can be measured [3]. Surprisingly, the magnitude of the response of T cells derived from mangabeys appears to be consistently lower than the proliferative response of cells derived from experimentally infected rhesus macaques. These findings prompted us to further examine the immune response to SIV in these two species at the level of individual viral proteins. In terms of the humoral immune response, this comparison is easily facilitated by Western blot analysis; however, such an assay only tangentially determines what is taking place at the cellular level. This is particularly true in HIV infection since HIV is known to polyclonally activate B cells [9]. As such, a humoral response in the absence of antigen-specific T-helper cells is not inconceivable. Thus, in order to examine the cellular immune response to individual viral proteins, we have developed the cellular Western blot assay. In this technique, viral proteins are separated by gel electrophoresis and transferred to nitrocellulose; bands are then cut out and used as antigen in the proliferation assay. This assay and preliminary results comparing the response of cells derived from mangabeys and rhesus form the basis of this report.

MATERIALS AND METHODS

Animals

Adult rhesus macaques (*Macaca mulatta*) and sooty mangabeys (*Cercocebus atys*) housed at YRPRC were the source of blood samples used in this study. Rhesus macaques were experimentally injected with 10^4 TCID of SIV/SMM as previously described [6] while the seropositive mangabeys used in this study were all naturally infected with SIV/SMM.

Isolation of Lymphocytes and Antigen-Presenting Cells (ACPs)

PBMC were isolated over a 60% Percoll gradient as previously described [2]. PBMC were then cultured at a concentration of 5×10^6 cells/ml in 7×7 cm polystyrene petri dishes (Corning Glass, New York, NY) at 37°C in a 7% CO_2 humidified atmosphere incubator for one hour. Nonadherent cells were aspirated, the petri dishes were placed on ice for 15 minutes, and adherent cells were removed with the help of a cell scraper. Both nonadherent and adherent cells were washed with media and viable cell counts determined using trypan blue exclusion. Adherent cells were adjusted to 10^6 cells/ml while nonadherent cells were adjusted to 5×10^6 cells/ml.

Separation of Viral Proteins

A pool of SIV/SMM was grown in the HT cell line and commercially prepared by contract (Advanced Biotechnologies, Silver Spring, MD) for our laboratory. The pooled $2000 \times$ concentrated virus contained 5×10^{10} virus particles/ml and was aliquoted and stored at -196°C . The virus preparation was uv inactivated as previously described [3].

For standard Western blot analysis, $1.5 \mu\text{g}$ of virus/well was separated on a 10% polyacrylamide gel and transferred to nitrocellulose, using the methods described for the BIORAD Western blot kit. Fig. 1 shows how this technique was modified for the cellular Western blot assay. Lanes 1, 5, and 9 consist of molecular weight markers. Lanes 2, 4, 6, and 8 are imaged using a total protein stain and act as templates for aseptically excising the protein bands. Lanes 3 and 7 are wide lanes (each the equivalent of 5 wells) in which $115 \mu\text{g}$ of virus is separated and excised for the proliferation assay. After the proteins are transferred to nitrocellulose, the nitrocellulose is aseptically removed from the transfer apparatus. Lanes 3 and 7 are cut out, using a sterile scalpel blade, washed extensively with media, and stored at -20°C until they are used. The other lanes are stained with the total protein stain and lined up as shown in Fig. 1. On the day of assay, the bands denoted 1–6 are drawn based on the MW markers and the protein stain. A piece of sterile acetate (normally used for making overhead projections) is placed over the template, and lanes 3 and 7 are superimposed over the template in the appropriate positions in a laminar flow hood. Using a sterile scalpel blade, bands 1–6 are excised from lanes 3 and 7 and then further cut into pieces of 1 mm in length. This is facilitated by the fact that the nitrocellulose is translucent, and the bands from the template may easily be traced by the scalpel blade.

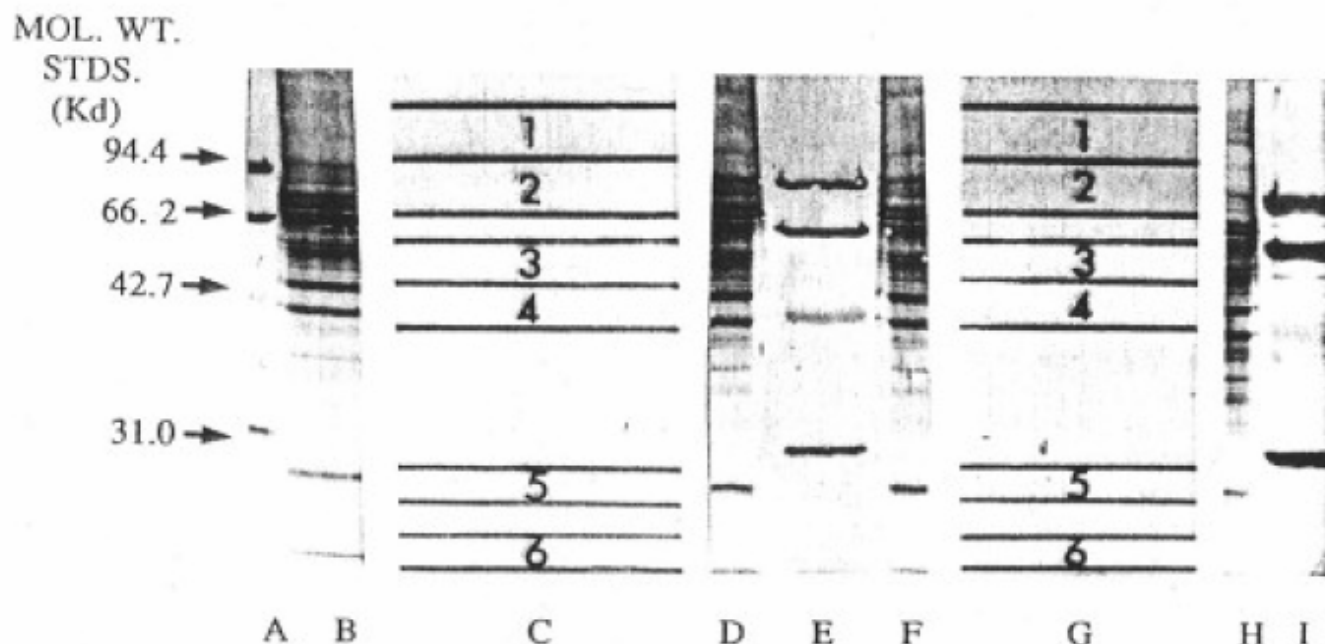


Fig. 1. Template for cellular Western blot assay. Using the molecular weight markers (lanes A, E, I) and total protein stained virus (lanes B, D, F, H) as a guide, bands 1–6 are aseptically excised from the unstained wide lanes 3 and 7. The bands are further spliced into 1 mm pieces and used as antigen in the proliferation assay.

Proliferation Assay

The proliferation of T cells to SIV/SMM has previously been described [3]. For the cellular Western blot assay, 10^5 APCs are incubated at 37°C in a 7% CO_2 atmosphere for three hours with a 1 mm piece of nitrocellulose from each of the protein bands, followed by an additional 1 mm piece of sterile nitrocellulose. It is important to place the additional sterile nitrocellulose piece after adding the APCs in order to retain them in the microtiter wells. All procedures are performed in the laminar flow hood. Next, 5×10^5 nonadherent cells are added, and the plates are incubated at 37° in a 7% CO_2 humidified atmosphere for five days, at which time the wells are pulsed with 1 μCi of methyl- ^3H -thymidine (^3H -TdR, specific activity 2 Ci/mM, NEN, Boston, MA) in 20 μl of media and harvested on day 6 with a SKATRON harvester. The mean counts per minute (cpm) of triplicate samples were determined by liquid scintillation spectrometry, using an LKB beta counter. The stimulation index (SI) was calculated by dividing the mean cpm of experimental cultures by the mean cpm obtained with the nitrocellulose blank. All assays were performed in triplicate, and results are expressed as the mean cpm \pm the standard deviation (SD).

Media

The media used for this assay was RPMI 1640 supplemented with 100 units/ml of penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (all from Gibco, Grand Island, NY). Positive controls for the proliferation assay consisted of media supplemented with rIL-2, a kind gift from Roche Laboratories, Nutley, NJ, at a final concentration of 500 units/ml.

RESULTS

Western Blot Analysis

Both naturally infected sooty mangabeys and experimentally infected rhesus macaques demonstrate a marked humoral response to SIV as determined by the presence of SIV/SMM-specific serum antibodies. Thus, we wanted to determine whether or not the antibodies in the serum of each species recognize the same viral proteins. Fig. 2 is representative of a typical Western blot comparing the humoral response of the two species. As seen in Fig. 2, both mangabeys and rhesus appear to make antibodies to the same viral proteins. There is some variability between the response of each animal. However, none of the proteins appears to be particularly immunogenic for one species and not the other. On the other hand, these data do not indicate differences, if any, that may be present in the epitope of each protein of SIV/SMM recognized by each species.

Cellular Western Blot Analysis in Mangabeys

Bands 1–6, prepared as described in the Materials and Methods section, were used as antigen in the proliferation assay. As seen in Table I, T cells from a seropositive mangabey proliferate strongly to bands 4, 5, and 6, with approximate molecular weights of 40, 25, and 15 kilodaltons (kd) respectively. The lack of a proliferative response to viral proteins of high molecular weight is somewhat surprising; however, it is not clear whether this represents a real phenomenon or whether this observation is due to a technical difficulty with the assay. It is clear that mangabeys make antibody to these bands (see Fig. 2). Thus, it is unlikely that the lack of response observed is due to insufficient antigen. There is no proliferative response to the nitrocellulose alone. In fact, the proliferative response of the negative controls is extremely low, suggesting that the nitrocellulose may cause some non-

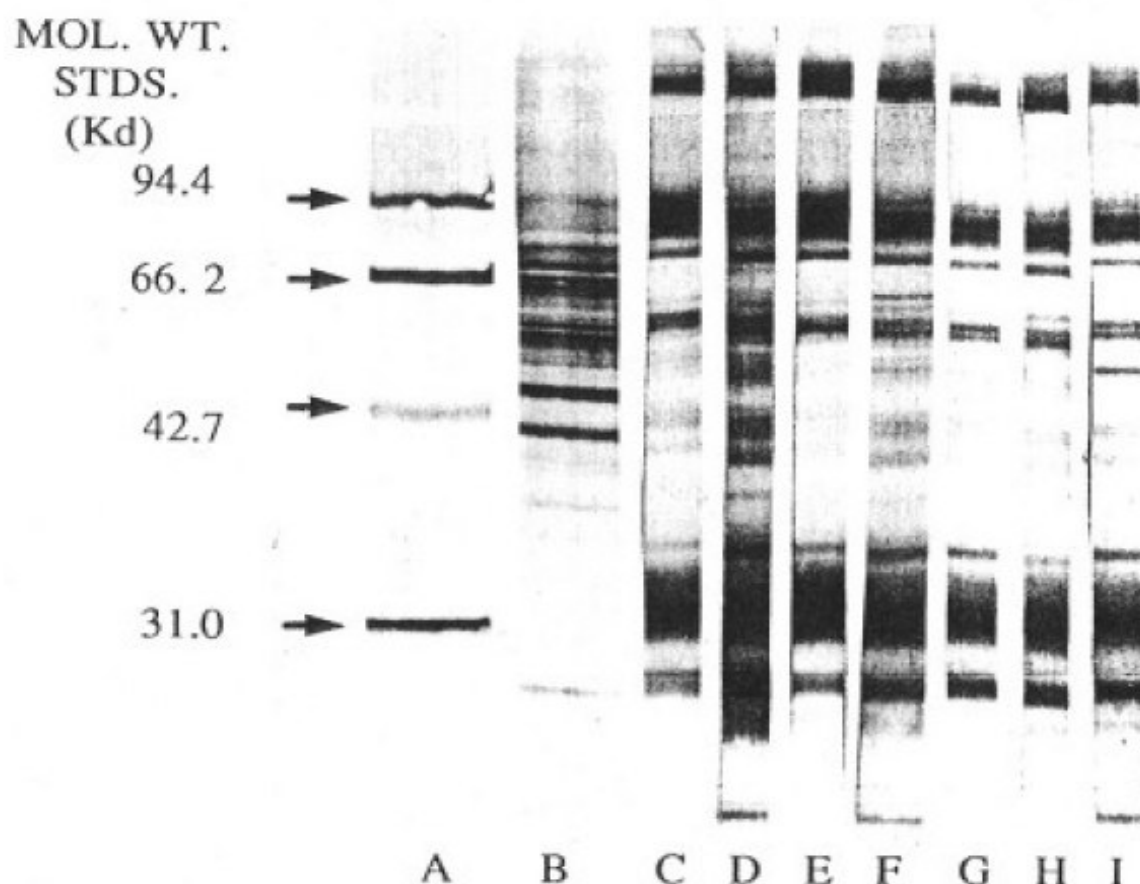


Fig. 2. Western blot of serum from mangabeys and rhesus. This is a standard Western blot in order to demonstrate SIV-specific antibodies in the serum of four naturally infected sooty mangabeys (lanes C, D, E, F) and three experimentally infected rhesus macaques (lane G, H, I). In lane A are the molecular weight markers, and in lane B is a total protein stain of the virus. Note that there is variation in terms of the protein-specific antibodies found in the serum of each of the monkeys; however, there is no apparent difference between the antibodies found in the serum of mangabeys and rhesus.

specific inhibition. Moreover, the cells from the seronegative mangabey fail to respond to any of the bands although they proliferate strongly in the presence of IL-2. This is an important control in that it suggests that the proliferative response is SIV specific and not due to contaminating proteins in the virus preparation.

During the course of our studies, we found that there was limited or no proliferative response in this assay using cells from a number of seropositive mangabeys. Such findings prompted us to examine the kinetics of the response for cells from mangabeys. The results of one such experiment are shown in Table II. As seen in Table II, there was a marked proliferative response on day 3, which had disappeared by day 5. Currently, experiments

TABLE I. Cellular Western blot assay for seropositive and seronegative mangabeys

Band	Relative MW	Mangabey (-)		Mangabey (+)	
		cpm ¹	SI ²	cpm	SI
Blank	—	386.0 ± 15.8	—	443.8 ± 93.0	—
1	100 × 10 ³	725.0 ± 14.1	1.9	766.0 ± 264.0	1.7
2	80 × 10 ³	541.3 ± 66.0	1.4	666.5 ± 90.0	1.5
3	50 × 10 ³	630.0 ± 99.0	1.6	1,543.0 ± 1,111.0	3.5
4	40 × 10 ³	663.6 ± 102.0	1.7	16,989.0 ± 3,448.0	38.2
5	25 × 10 ³	456.7 ± 55.0	1.2	13,247.0 ± 3,051.0	29.8
6	15 × 10 ³	671.7 ± 15.7	1.7	9,709.0 ± 345.0	21.9
rIL-2	—	38,161.0 ± 2,719.0	100.0	37,787.0 ± 1,100.0	78.4

¹Proliferative response in counts per minute (cpm) ± standard deviation.

²Stimulation index (SI) was calculated by dividing proliferative response by the baseline proliferation of the blank.

are in progress to investigate the possibility that perhaps in these mangabeys, the proliferative response is primarily by CD8⁺ cells, which, in turn, suppress the latter response. This hypothesis is supported to some extent by our experience in measuring the proliferative response to whole SIV/SMM in mangabeys, which consistently proliferate to a lesser extent to whole SIV/SMM than cells from infected rhesus macaques.

Cellular Western Blot Analysis in Rhesus Macaques

As was demonstrated for the mangabeys, the proliferative response of the T cells from infected rhesus macaques was also directed against the proteins of lower molecular weight. As was the case with whole SIV/SMM, in general, the proliferation of cells from infected rhesus macaques was higher than that of cells from seropositive mangabeys. This is dramatically illustrated in Table III. Once again, note that there is no response by the cells derived from the uninfected rhesus macaque. Moreover, whereas cells from rhesus 1 respond to band 6, cells from rhesus 2 do not respond to this protein. Approximately two weeks later, T cells from both of these monkeys showed a similar in vitro proliferative pattern although the magnitude of the response was decreased by 1 log. In addition, it is interesting to note that rhesus 1 died approximately two months after this assay was performed. Of interest is the observation that the antigen-specific proliferative response of T cells from SIV-infected rhesus macaques always peaked on day 5, unlike T cells from SIV-seropositive sooty mangabeys, which showed peak proliferative response on day 3.

TABLE II. Kinetics of the cellular Western blot assay for a seropositive mangabey

Band	Relative MW	SI ¹ day 3 ²	SI day 5
3	50 × 10 ³	< 1	1.0
4	40 × 10 ³	51.1	5.5
5	25 × 10 ³	33.3	9.6
6	15 × 10 ³	28.7	< 1
rIL-2	—	31.1	365.5

¹ Stimulation index (SI) was calculated by dividing proliferative response by the baseline proliferation of the blank.

² Day 3 cultures were pulsed with radioactive thymidine on day 2 and harvested on day 3 while day 5 cultures were pulsed on day 4 and harvested on day 5.

DISCUSSION

Previously, our laboratory has described a proliferation assay which measures the SIV/SMM antigen-specific T cell response of experimentally infected rhesus macaques and naturally infected mangabeys [3]. In this communication we report our preliminary findings concerning the cellular Western blot assay. Unlike our whole virus assay, the cellular Western blot assay facilitates the examination of the proliferative response to individual viral proteins. T cells from both SIV/SMM infected rhesus and mangabeys respond well to viral proteins of approximately 40, 25, and 15 kd molecular weight. These proteins are most likely to be part of *gag* and envelop breakdown products. However, with the development of monoclonal antibody reagents to SIV, we hope to be more definitive about the nature of these proteins. The response being measured is not a non-specific response to cellular debris which has contaminated our viral preparations because proliferation to these bands using cells from uninfected rhesus has never been demonstrated.

Conventional Western blot analysis of the serum of seropositive mangabeys and seropositive rhesus does not show any distinct differences between the humoral response to SIV between these two species. Likewise, the present data fail to demonstrate any differential response to these protein bands in terms of the cellular immune response. However, the response of cells for SIV/SMM infected rhesus macaques was, in general, of a greater magnitude, and initial data suggest that the kinetics of the proliferative response is different for the two species. Both of these observations suggest that the response in the mangabeys may involve the preferential expansion of

TABLE III. Cellular Western blot assay for infected and uninfected rhesus

Band	Relative MW	Rhesus (-)		Rhesus 1 (+)		Rhesus 2 (+)	
		cpm ¹	SI ²	cpm	SI	cpm	SI
Blank	-	131.0 ± 19.0	-	77.5 ± 27.3	-	55.2 ± 29.0	-
4	40 × 10 ³	93.6 ± 22.0	< 1	234,406 ± 2.4 × 10 ⁴	3.0 × 10 ³	241,979 ± 2.2 × 10 ⁴	4.4 × 10 ³
5	25 × 10 ³	134.0 ± 37.5	1.0	31,723 ± 7.1 × 10 ³	4.1 × 10 ²	23,931 ± 1.0 × 10 ³	4.3 × 10 ²
6	15 × 10 ³	84.9 ± 38.8	< 1	13,765 ± 4.5 × 10 ³	1.8 × 10 ³	38.9 ± 9.1	< 1

¹ Proliferative response in counts per minute (cpm) ± standard deviation.² Stimulation index (SI) was calculated by dividing proliferative response by the baseline proliferation of the blank.

CD8⁺ suppressor cells. Along these lines, we have been able to easily isolate CD8⁺ SIV-specific T cell clones from mangabeys, using our whole virus proliferation assay. Currently, we are attempting to define the subsets responsible for the proliferative response in this assay by depleting PBMC of T cell subsets and blocking with monoclonal reagents directed against Class I and Class II MHC molecules.

Thus, even though T cells from the mangabeys and the rhesus macaques respond to the same protein bands, both the quality and the quantity of the response appears to differ between the two species. It is of interest to note that the vigorous responses seen in Table III occurred at a time when both rhesus were showing signs of immunosuppressive disease. The first rhesus ultimately died whereas the second rhesus has since stabilized. In addition, in subsequent experiments using cells from this animal, the magnitude of the response in the second rhesus has markedly decreased (data not shown). Perhaps such data is indicative of pathogenic immunity.

Initially, we had hoped to utilize this assay to sequentially monitor the humoral and cellular response to viral peptides during the course of infection in experimentally infected rhesus macaques. In this regard, it was thought that the cellular Western blot assay might detect immune deficiencies at the cellular level before there was a concomitant loss of antibody to a particular viral protein. For example, it is known that the antibody response to *gag* is often lost immediately prior to the death of the animal. With the use of the cellular Western blot assay, it might be shown that the T-helper cell response to *gag* is lost in advance of any detectable loss of antibody. This assay was very consistent in that in limited sequential studies on two infected rhesus macaques for a period of two months, the PBMC from these animals always responded to the same bands. The lack of reactivity to high molecular weight proteins is disturbing. It may be due to lack of optimal concentration of the protein or due to denaturation of the protein in a form not optimal for antigen processing or presentation. It is also possible that non-specific inhibitors previously reported to occur in HIV and SIV preparations may be present in these high molecular weight bands. Certainly, more detailed studies are warranted in efforts to explore this novel technique for the delineation of peptide-specific immunity in SIV-infected animals.

The cellular Western blot assay offers a unique strategy for defining immunogenic epitopes. Band 4 appears to be exceptionally immunogenic for both mangabey and rhesus T cells. In the future the objective will be to microelute this protein from the nitrocellulose and enzymatically digest it [1]. The peptides derived from the protein will be isolated by HPLC. Each

fraction will be tested in our proliferation assay, and those that give a positive response will be sequenced. In this way, immunogenic peptides can be isolated and selected according to the immune response of the animals. Ultimately, the fine dissection of immune response at the epitope level may show profound differences between the responses of experimentally infected rhesus macaques and naturally infected sooty mangabeys. In this regard, the consistently lower response seen in mangabeys may be due to lack of recognition of certain epitopes on a viral protein. Furthermore, an immune response to such epitopes might be associated with pathogenesis as opposed to protection. In addition, such strategies may be used to define differential humoral responses between the two species at the epitope level.

In summary, we have described a novel approach to examine the cellular immune response to SIV viral peptides. In the future we hope to utilize this assay in order to compare the humoral and cellular immune response to particular viral proteins and to compare the immune response of SIV infected mangabeys and rhesus macaques. In addition, we believe that this assay provides a unique strategy for defining immunogenic viral epitopes.

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