Immunology 1986 **57** 437–442

Human immune responses to herpes simplex virus, varicella-zoster and cytomegalovirus *in vitro*

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Accepted for publication 22 November 1985

SUMMARY

The cell principally responsible for lymphocyte proliferation to herpes simplex virus (HSV), varicella-zoster (VZ) and cytomegalovirus (CMV) has been shown to be a T cell of helper phenotype. Lymphocytes from a proportion of proliferation-positive normal individuals produced anti-viral antibody *in vitro*. Although in some cases, and at some time-points, the antibody was specific for the priming virus, in others, antibodies to more than one virus were detected. Similarly, some T-cell clones proliferated specifically to the priming virus, whereas others were not specific for the virus used in the priming culture. Two clones helped the production of HSV-specific antibody, one by autologous, the other by both autologous and allogeneic non-T cells.

INTRODUCTION

Viruses of the herpes group often become latent following the primary infection. The fact that in immunosuppressed patients viral reactivation is common, and leads to particularly severe disease, suggests that immunological mechanisms play an important role in the maintenance of the latent state.

In man, it is well established that circulating specific antibody titres rise following infection with HSV and VZ viruses (Schmidt, Lennett & Magoffin, 1969) and with CMV (The & Langenhuysen, 1972). Roughly 70% of CMV seropositive normal adult donors also had significant lymphoproliferation to CMV, although all individuals who had antibodies to CMV early antigens were proliferation-positive (Ten Napel *et al.*, 1977). Lymphocytes from normal adults also proliferate to HSV (Ten Napel *et al.*, 1977). In patients with recurrent HSV infection, there is also some suggestion of a time relationship between the magnitude of the lymphoproliferative response to both envelope and capsid antigens and the severity of clinical symptoms (Kalimo, Joronen & Havu, 1983). The production of *in vitro* specific antibodies to VZ (Souhami, Babbage & Callard, 1981) and HSV (Souhami, Babbage & Sigfusson, 1983) has also been described.

Herpes virus specific cytotoxic cells have also been described in man. For example, Quinnan *et al.* (1981) have shown that CMV-specific cytotoxic T and non-T cells develop during CMV reactivation in bone marrow transplant recipients, the cytotoxic T cells in particular showing evidence of MHC restriction. Human cytotoxic T-cell clones, which discriminate between HSV-1 and HSV-2 infected target cells, are of helper phenotype, and are restricted by MHC class II antigens (Yasukawa & Zarling, 1984a, b).

In the mouse, it has been possible to evaluate the contribution of different immune mechanisms to protection against lethal HSV-1 infection. Passive immunization with monoclonal antibodies to the glycoproteins gB, gC, gD and gE confers varying degrees of protection (Dix, Pereira & Baringer, 1981; Roberts *et al.*, 1985), although the protective effect of active immunization with affinity-purified glycoproteins is more impressive (Roberts *et al.*, 1985). Two different types of protective T cell have been described. Protection against intraperitoneal challenge has been achieved by transfer of Lyt 2+, MHC class I-restricted, cytotoxic T-cell clones (Sethi, Omata & Schneweis, 1983). However, in adoptive transfer experiments in sublethally irradiated mice challenged intradermally or subcutaneously, protection was mostly due to an Lyt 2−, MHC class II-restricted T cell (Nash & Gell, 1983).

In mice inoculated intradermally or subcutaneously, therefore, there is a contrast between the class II-restricted protective response to HSV, and the class I-restricted protective response to non-latent viruses such as influenza (Yap, Ada & Mckenzie, 1978). There is a corresponding contrast in man between class II-restricted cytotoxic T-cell clones specific for HSV (Yasukawa & Zarling, 1984a) and class I-restricted influenza specific cytotoxic T cells (McMichael *et al.*, 1980). However, whereas *in vitro* antibody responses to influenza virus have been well studied in man (Callard, 1979; Callard & Smith, 1981; Eckels *et al.*, 1983; Lamb *et al.*, 1982a, b, c), there is little information on human *in vitro* antibody responses to herpes viruses.

In this paper, we show that lymphocytes from a proportion of normal individuals produce specific anti-HSV and anti-CMV antibodies *in vitro*, and describe a T-cell clone that provides specific help for anti-HSV antibody production.

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MATERIALS AND METHODS

Antigens

Viral and control antigens were obtained from Tissue Culture Services (TCS, Slough, Berks) as lyophilized powders. CMV and VZ viruses were grown in human fibroblasts, and an extract of uninfected human fibroblasts (HFA) was used as control antigen. In the case of HSV, similarly, the control antigen was non-infected Vero cells (Vero). Each vial of antigen was reconstituted in 2 ml of RPMI (RPMI-1640 powder from Flow Laboratories, Irvine, Ayrshire, supplemented with 10 mM HEPES, 1·65 g/l sodium bicarbonate, 2×10^5 IU/l penicillin and 0·1 g/l streptomycin). The antigens were stored at 4° in 50-μl aliquots until used. The optimal concentrations of viral and control antigens for proliferation and *in vitro* antibody assays were determined in preliminary experiments.

Donors studied

Normal control individuals were healthy hospital staff. Post bone marrow transplant patients had received a bone marrow graft between 3 months and 5 years previously for inborn errors of metabolism, primary immunodeficiencies, leukaemia or aplasia. Although some patients were still receiving immunosuppressive therapy at the time of testing, all had mixed lymphocyte and candida responses within the normal range. In addition, a group of nine patients with recurrent genital herpes was studied.

Separation of peripheral blood mononuclear cells

Peripheral blood was collected into lithium heparin tubes (Sterilin, Teddington, Middlesex). The plasma was removed after centrifugation and the blood was reconstituted with RPMI. Peripheral blood mononuclear cells (PBMs) were separated on sodium metrizoate (density 1·086 g/ml, Lymphopaque, Nyegaard, Oslo, Norway) by centrifugation at 800 *g* for 30 min. PBMs were washed three times before being used in any assay.

Proliferation assays

The proliferation assay of fresh PBMs was performed essentially as described by Ten Napel & The (1980). Briefly, 10^5 PBMs were cultured in RPMI containing 25% autologous plasma and a previously determined optimum concentration of antigen in flat-bottomed 96-well microtitre plates (Nunc, Gibco, Uxbridge, Middlesex) for 5 days. Peak proliferation by PBMs from proliferation-positive individuals had been shown previously to occur at 5 days, and varying the culture time from 3 to 10 days with PBMs from proliferation-negative donors did not reveal a response. The plates were pulsed with 0·5 μCi [^{3}H]thymidine (TRK61, Radiochemical Centre, Amersham, Bucks, specific activity approximately 23 Ci/mmol) for 18 hr prior to being harvested onto glass fibre discs. After drying, these were counted in a liquid scintillation counter (Rackbeta, Model 1212, LKB, Bromma, Sweden). A positive response was one where the incremental c.p.m. (virus − control antigen) was greater than control c.p.m. + 2 SD and exceeded 2000 c.p.m. Proliferation of T-cell clones was assayed similarly, except that 1×10^4 responding cloned T cells with $2{\cdot}5 \times 10^4$ autologous or allogeneic irradiated (3000 rads) PBMs were incubated for 3 days in 10% autologous plasma in U-bottomed microtitre plates (Nunc).

Cell sorting

Cell sorting was performed on a FACS 420 (Becton-Dickinson, Mountain View, CA). PBMs were separated into E-rosette forming (E +ve) cells and non-rosetting (E −ve) cells using sheep red blood cells (SRBC) treated with 2-aminoethylisothiouronium bromide hydrobromide (AET Sigma) as described by Kaplan & Clarke (1974). E +ve and E −ve populations were separated on Percoll (Sigma) at a density of 1·087 g/ml. The E −ve cells were recovered from the interface and the E +ve from the pellet after lysis of the SRBC with sterile water and immediate dilution with RPMI. The E +ve cells were washed twice and resuspended at approximately 10^7 cells/ml in a 1:80 dilution of the monoclonal antibody (Leu-3a or Leu-2a, from Becton-Dickinson) and kept on ice for 15 min. After a further wash in cold RPMI, the E +ve cells were resuspended in a 1:80 dilution of a F(ab′)$_2$ fragment of goat anti-mouse IgG FITC (Cappell Laboratories, Dynatech, Cochranville, PA) for a further 15 min and kept on ice. After two washes, the cells were resuspended at 1×10^6 cells/ml in cold RPMI and kept on ice until sorted. The E −ve cells were given 3000 rads irradiation (from a ^{137}Cs source, Gammacell 1000, Atomic Energy of Canada, Ottawa) before being used. Proliferation assays were set up as described above using 1×10^5 E +ve cells per well and 1×10^3 E −ve cells per well.

IL-2 production

IL-2 was produced as previously described by Lamb *et al.* (1982a), using 2·5 μg/ml PHA-P (Wellcome HA16) and 10% pooled plasma.

Cloning of T lymphocytes

Production of T-cell clones to viral antigen has been described previously by Lamb *et al.* (1982a). Briefly, PBMs were stimulated at $1{\cdot}5 \times 10^6$ cells/ml in RPMI supplemented with 10% autologous plasma and optimum antigen concentration. After 6 days, the responding lymphoblasts were cloned by limiting dilution at 0·6 cells per well in Terasaki micro plates in 20 μl of the above medium containing 25% IL-2 and 5×10^5/ml autologous irradiated feeder cells. The cultures were expanded as described by Lamb *et al.* (1982a).

In vitro *antibody production*

In vitro antibody responses were made in Falcon 2054 tubes as described by Callard & Smith (1981) with the following modifications: 3×10^6 PBMs per tube in 10% fetal calf serum (FCS) instead of 10% horse serum, and a 1:2000 dilution of viral antigen.

ELISA assay

Specific anti-viral antibodies in plasma and supernatants were assayed by an ELISA technique. Optimal concentrations of control and viral antigens were absorbed onto flat-bottomed microtitre plates (Virion A, Lodge Diagnostics, Clydebank, Strathclyde) in 0·05 M carbonate buffer, pH 9·6, at 4° overnight. The plates were washed in phosphate-buffered saline containing 1% Tween 20, and residual sites were blocked with 2% bovine serum albumin. Dilutions of plasma, or neat supernatants, were incubated in triplicate in the wells for 2 hr at room temperature. After a further wash, an optimum dilution of peroxidase-labelled rabbit anti-human IgG (Dako, Weybridge, Surrey) was added to the wells. The plates were washed again, and the wells

Table 1. Lymphocyte proliferation in response to herpes viruses

	% proliferation positive to VZ	% proliferation positive to HSV	% proliferation positive to CMV
Normals	77% (30)	53% (34)	45·5% (33)
Genital herpes patients	NT	100% (9)	NT
Post-BMT patients	40% (10)	60% (15)	19% (31)

Numbers tested are given in parentheses.
NT, not tested.

incubated with 3,3′5,5′-tetramethylbenzidine (Aldrich Chemical Company, Milwaukee, WI) in 0·5 M sodium citrate buffer containing hydrogen peroxide. The reaction was stopped after 1 hr by the addition of 12·5% (v/v) sulphuric acid, and the absorbance at 450 nm read in a Titertek Multiscan Elisa Reader.

Results are expressed as absorbance to viral antigen − absorbance to control antigen (incremental absorbance). Absorbance to control antigens did not exceed 0·15 for supernatants or 0·25 for 1:1000 or greater dilutions of plasma. For an incremental response to be considered significant, absorbance to viral antigen − 1 SD has to exceed absorbance to control antigen + 1 SD, and the magnitude of the incremental absorbance has to exceed 0·15.

Helper assay

Help for antibody production by the T-cell clones was assessed by culturing 1×10^4 cloned cells/tube with 5×10^5 E −ve cells in 1 ml with antigen as described above.

RESULTS

Proliferation responses

Lymphocytes from between 45% and 77% of normal individuals proliferated to at least one of the three herpes viruses used in the present study (Table 1). Some donors did not respond to any virus, others responded to one, two or three of the viruses. In post-BMT patients, there is insufficient data to evaluate whether the differences in the incidence of responses to VZ or CMV are real. Lymphocytes from all nine patients with recurrent genital herpes showed significant proliferation to HSV.

In normal donors, the responding cell in the proliferation assay was predominantly of helper phenotype; a representative experiment using HSV as antigen is shown in Fig. 1. Similar results were obtained using VZ and CMV. In all experiments there was a clear requirement for E −ve antigen-presenting cells (APC). Although cells of helper phenotype (Leu-3a +ve, Leu-2a −ve) showed the stronger proliferation, in most experiments a lower, but still significant, response was seen with cells of suppressor/cytotoxic phenotype (Leu-3a −ve, Leu-2a +ve). This pattern was similar, irrespective of antigen dose, and whether cultures were pulsed at 3, 5 or 10 days.

Antibody production *in vitro*

A proportion of normal donors whose lymphocytes proliferated to either HSV or CMV produced specific anti-HSV or anti-CMV antibodies *in vitro*. For example, 6/13 (46%) HSV proliferation-positive donors produced specific IgG antibody. Similarly, specific anti-CMV antibody was made by cells from 3/18 (17%) CMV proliferation-positive donors. Representative examples are shown in Fig. 2a for HSV and in Fig. 2b for CMV.

In Fig. 2a, the first donor produced antibodies to VZ as well as to HSV on Day 7 of culture; IgG produced by the second donor at this time was HSV-specific. Neither individual produced anti-CMV antibodies when their cells were stimulated with HSV. Lymphocytes from both of these donors proliferated

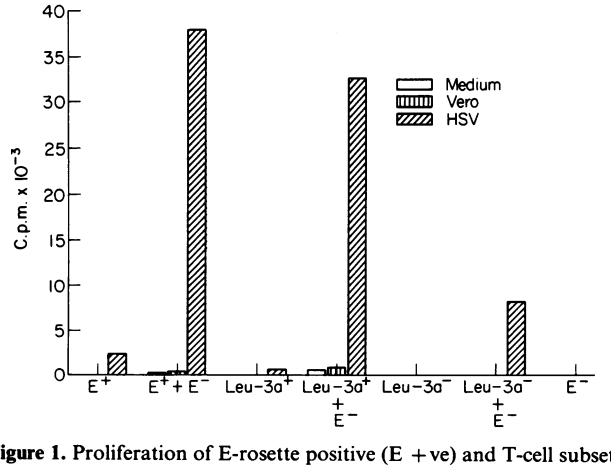

Figure 1. Proliferation of E-rosette positive (E +ve) and T-cell subsets to HSV and control antigens, with and without autologous E-rosette negative (E −ve) cells. All E −ve cells had been irradiated with 3000 rads.

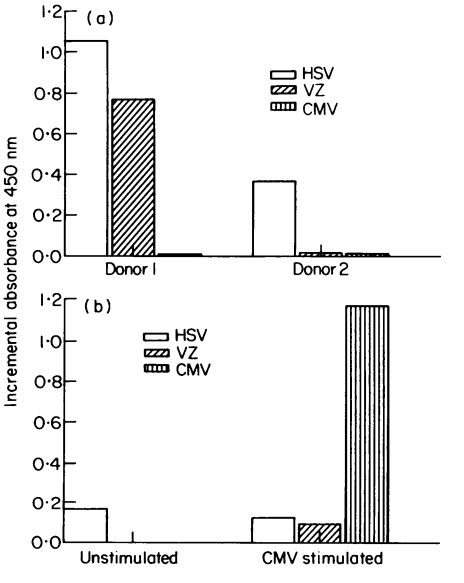

Figure 2. (a) Production of antibodies to HSV, VZ and CMV *in vitro* by HSV stimulated lymphocytes from two donors whose lymphocytes proliferated to HSV and VZ. Supernatants were assayed after 7 days of culture. (b) Production of antibodies to HSV, VZ and CMV *in vitro* by CMV stimulated and unstimulated lymphocytes from a single donor whose lymphocytes proliferated to CMV and VZ. Supernatants were assayed after 7 days of culture.

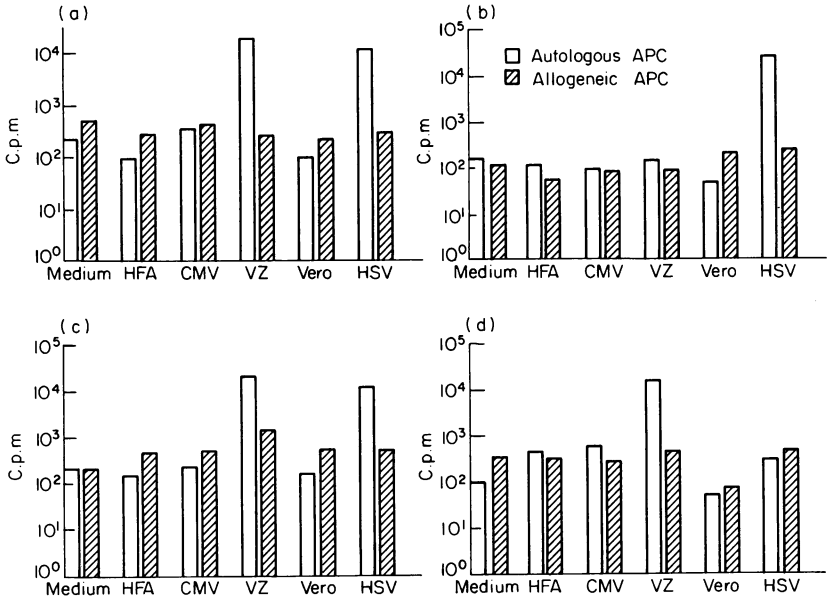

Figure 3. Proliferation to viral and control antigens by four T-cell clones in the presence of autologous or allogeneic irradiated (3000 rads) PBMs. Cultures were pulsed on Day 3. Two HSV primed clones are shown in (a) and (b) [HA8, (a); HA11, (b)] and two VZ primed clones in (c) and (d) [VB10, (c); VC6, (d)].

to HSV and VZ but not to CMV. The single donor shown in Fig. 2b produced CMV-specific antibodies. In this experiment, the incremental absorbance to HSV by supernatant from unstimulated cells, and to HSV and VZ by supernatant from stimulated cultures, was not significant.

There was a tendency, irrespective of which virus was used as the immunogen, for the *in vitro* response to lose specificity at later time-points. Thus, the second donor in Fig. 2a produced anti-VZ as well as anti-HSV antibodies by Day 9 of culture. Similarly, the donor in Fig. 2b produced anti-VZ as well as anti-CMV antibodies by Day 9.

Proliferation of T-cell clones

T cells were cloned from proliferation mass cultures stimulated with either HSV, VZ or CMV. All the clones described here were consistently of helper phenotype. Many clones, although showing MHC restriction, were not specific for the stimulating virus. Some representative examples are shown in Fig. 3. Thus, of two clones primed to HSV, one (HA11, Fig. 3b) was HSV-specific, the other (HA8, Fig. 3a) proliferated to both HSV and VZ. Similarly, some VZ-primed clones proliferated specifically to VZ (e.g. VC6, Fig. 3d), whereas others (e.g. VB10, Fig. 3c) responded to both VZ and HSV. These patterns of response were maintained over roughly an eight-fold antigen concentration range, and persisted for periods of at least 9 months in culture. We have raised 12 clones that proliferated specifically to the stimulating virus. A further 10 reacted with both the stimulating and at least one other virus.

In the case of one specific (HA11) and one non-specific clone (VB10), the patterns of reactivity were retained in subclones derived from the parent culture by limiting dilution. In particular, it has not been possible, by subcloning, to separate response to one virus from response to another in non-specific clones such as VB10.

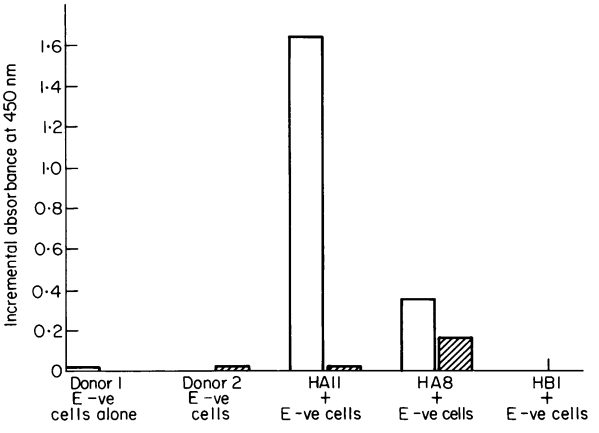

Figure 4. Help for anti-HSV antibody production by autologous and allogeneic E −ve cells from HSV primed T-cell clones.

Clonal help

The T-cell clones were tested for their ability to help autologous and allogeneic E −ve cells produce viral specific IgG antibodies. In the experiment shown in Fig. 4, fresh E −ve cells from neither donor produced HSV-specific antibody without T-cell help. When fresh autologous E +ve cells were added, Donors 1 and 2 produced HSV-specific IgG which gave absorbances of 0·516 and 1·342, respectively. The helper activities of three clones, all derived from Donor 1, and all of helper phenotype, are shown in Fig. 4. Clearly, clone HA11 only helps autologous E −ve cells. Clone HA8, however, consistently helps both autologous and allogeneic E −ve cells, the incremental absorbance of 0·16 with allogeneic E −ve cells in the experiment shown conforming to the criteria of significance described in the Materials and Methods. Neither autologous nor allogeneic E −ve cells are

helped by clone HB1. None of these supernatants, from both cultures containing fresh and cloned T cells, contained anti-VZ antibody.

DISCUSSION

In this paper we have described some aspects of the human immune response to herpes viruses. Studies of the specificity of antibodies produced *in vitro*, and of the proliferation of T-cell clones, support the molecular evidence of extensive cross-reactivity between HSV, VZ and CMV (reviewed in Honess, 1984). Nevertheless, one T-cell clone has been identified that provides specific, MHC-restricted help for anti-HSV antibody production.

The proportion of normal individuals whose lymphocytes proliferate to CMV is similar to that reported in previous studies (Ten Napel *et al.*, 1977), although the percentage of donors responsive to HSV is rather lower. All donors whose lymphocytes proliferated to a given virus had virus-specific plasma antibody titres (measured by ELISA) of greater than 1:1000, but not all seropositive individuals showed specific lymphoproliferation. Proliferation was mostly due to T cells of helper phenotype (Fig. 1). Although we have not defined the restriction element in our system, either in mass culture or using T-cell clones, other workers have shown that virus-specific proliferation is restricted by the HLA-D region (Berle & Thorsby, 1980; Eckels *et al.*, 1983; Yasukawa & Zarling, 1984a, b).

Cells from all patients with recurrent genital herpes showed significant proliferation to HSV, a result in agreement with previous studies on patients with both genital and labial HSV infections (Kalimo *et al.*, 1983).

Lymphocytes from only a proportion of seropositive, T-cell proliferation-positive donors produced virus-specific antibody *in vitro*. The amount of total IgG in virus-stimulated culture supernatants was five to ten-fold less than in supernatants from pokeweed mitogen-stimulated cells (data not shown), and was comparable with the amount of influenza-specific IgG produced by influenza virus-stimulated lymphocytes (Callard, 1979). Although CMV has been reported to activate polyclonally B cells from CMV seronegative individuals (Hutt-Fletcher, Balachandran & Elkins, 1983), the data would suggest that antigen-specific activation is occurring in our system.

In studies on the decline of *in vitro* anti-influenza antibody production after vaccination, virus-specific T cells were shown to persist in the circulation longer than virus-specific non-T cells (Mitchell *et al.*, 1982). In our study, it may well be that those donors whose cells produced antibody *in vitro* had been exposed asymptomatically to virus antigen more recently than those whose cells did not. It is of interest that the ability to produce anti-HSV antibody *in vitro* appears to persist for longer periods (greater than 1 year) than *in vitro* production of anti-CMV antibody (less than 2 months).

Antibodies produced *in vitro* to one virus often also reacted with a different virus of the herpes group (Fig. 2a). Similarly, in proliferation assays, many T-cell clones responded to both the stimulating and at least one other virus, often in an MHC-restricted fashion. With HSV and VZ, it has been known for some time that serum antibody titres to one virus rise after infection with the other (Ross, Subak Sharpe & Ferry, 1965). More recently, Shiraki *et al.* (1982) have shown that non-neutralizing rabbit anti-HSV antibodies recognize a viral glycoprotein expressed on the nuclear membrane of VZ-infected cells. At the T-cell level, some OKT4 +ve cytotoxic T-cell clones recognize virus glycoproteins on both HSV-1 and HSV-2 infected target cells (Yasukawa & Zarling, 1984b).

Cloned T cells that help in influenza-specific antibody production in man have been extensively described (Eckels *et al.*, 1983; Lamb *et al.*, 1982a, b, c). These are invariably restricted by MHC class II antigens and some, although they help production of antibody specific for the viral envelope haemagglutinin, apparently recognize an internal virion component (Lamb *et al.*, 1982c). In this study, we describe two T-cell clones that help production of anti-HSV antibody *in vitro*, one (HA11) apparently restricted, the other (HA8) not. Although the number of specific helper clones we have raised is small, it may be significant that the anti-HSV response they assist does not cross-react with other herpes viruses.

ACKNOWLEDGMENTS

This work was supported by the Fane and Bostic Trusts. We are grateful to the patients and our colleagues for their generous donations of blood. We are particularly indebted to Professor J. R. Hobbs for his encouragement during these studies, and to both Professor Hobbs and Dr J. K. Oates for access to patients under their care.

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