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CORRELATION BETWEEN IMMUNOLOGICAL HYPERSENSITIVITY AND EPSTEIN-BARR VIRUS IN PATHOGENESIS OF CHRONIC EBV

THE UNIVERSITY OF ARIZONA M.S. 1984

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CORRELATION BETWEEN IMMUNOLOGICAL HYPERSENSITIVITY AND EPSTEIN-BARR VIRUS IN PATHOGENESIS OF CHRONIC EBV

by

Moien Nihad Kanan

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Date

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ABSTRACT

The purpose of this study was to compare the relationship between hypersensitivity disorders and the inability of the chronic EBV subjects to manage properly the EBV. Individuals from three groups were evaluated; EBNA-positive hypersensitive ill (chronic EBV), EBNA-positive hypersensitive healthy and EBNA-positive mild hypersensitive healthy subjects.

Studies revealed that subjects with chronic EBV: (1) have a higher rate of DNA synthesis in the nonstimulated PBL, (2) can be separated from other subjects with hypersensitivities on the basis of increased anti-VCA and anti-EA titers, (3) have stimulation indices against a battery of allergens with two to five fold greater than other EBNA-positive hypersensitive subjects, (4) have a greater percentage of EBNA-positive cells in PBL, and (5) develop a greater percentage of EBNA-positive cells following invitro stimulation with the culprit antigen than do cells obtained from other EBNA-positive hypersensitive subjects.

The data suggest that EBV can cause in certain individuals an abnormality not previously recognized.

CHAPTER 1

INTRODUCTION

Epstein-Barr Virus: Its Discovery and Significance

Epstein-Barr virus (EBV) was discovered in 1964. Since that time an immensity of information has been accumulated on this virus. The virus has very special relevance in human medicine and oncology, in tumor virology, in immunology and in molecular virology. It is the cause of infectious mononucleosis and is the first human cancer virus etiologically related to endemic Burkitts' lymphoma (BL) and nasopharyngeal carcinoma (NPC).

EBV is the most widely studied nonlytic herpesvirus. Herpesvirus types 1 and 2 produce typically cytopathic effects in many types of cell cultures, however, EBV infects only primate B lymphocytes and even in these cells the virus is only produced by an occasional infected cell (1).

EBV is transmitted horizontally and infects all human populations (2). Natural primary infection usually takes place in childhood without disease manifestations and it is always accompanied by permanent seroconversion and harboring of the virus for the rest of the individual's life (3). This persistence of virus is manifested, first

as a nonproductive latent infection of a small number of circulating lymphocytes, and second, as a productive infection somewhere in the nasopharynx with liberation of infectious virus into the buccal fluid. It is the infectious virus, shed into the buccal fluids, which is responsible, by horizontal transmission for all natural primary infections.

Natural primary infection, delayed until late young adulthood, will lead to infectious mononucleosis (IM) in about 50% of the cases (4). Delayed natural infections are more frequent in the privileged classes of developed societies than in the lower socioeconomic groups, thus explaining why IM is commonest among the affluent (5). In developing countries, primary infections affect almost all children before the age of 10, consequently, few young adults are susceptible and IM is virtually unknown (6).

EBV: Types of Cellular Infection and Virus Associated Antigens

Five different virus-determined antigens have been associated with virus infected cells. Four were discovered by the use of naturally occurring human antibodies, namely (1) EBV nuclear antigens (EBNA) (7), which is a DNA binding nonhistone-protein, (2) membrane antigen (MA) which has been subtyped into early (EMA) and late (LMA) components (8), (3) early antigen (EA) which has two subtypes, diffused

(D), and restricted (R) (9), and (4) viral capsid antigen (VCA). The fifth antigen is a lymphocyte detected membrane antigen (LYDMA), discovered in the course of invitro cytotoxicity tests using cytotoxicity T-cells specifically active against EBV genome-containing B-cells (10).

A proposed pathway for the replication of EBV in a productive infection is shown in Figure 1. This type of infection is accompanied by the expression of EBNA, LYDMA, EMA, EA, VCA, and LMA.

In addition to the productive infections, EBV can cause two types of nonproductive infections, these are the latent infections and the malignant transformations. In latent infections, the infectious virus will become apparent if the intact cells are cultured in vitro. The infectious virus is liberated into the medium where it infects and transforms B-cells (12). It is evident that the latently infected genome-containing B-cell must express LYDMA on the plasma membrane since IM cytotoxic T-cells specifically recognize this antigen (13).

The second type of nonproductive infection by EBV relates to malignant transformation. EBV will cause fatal, malignant reticulo-proliferative disease in cotton top marmosets and owl monkeys (14). In malignant transformation the virus will cause the expression of EBNA, LYMDA, and EMA. With the two human EBV-associated malignant tumors,

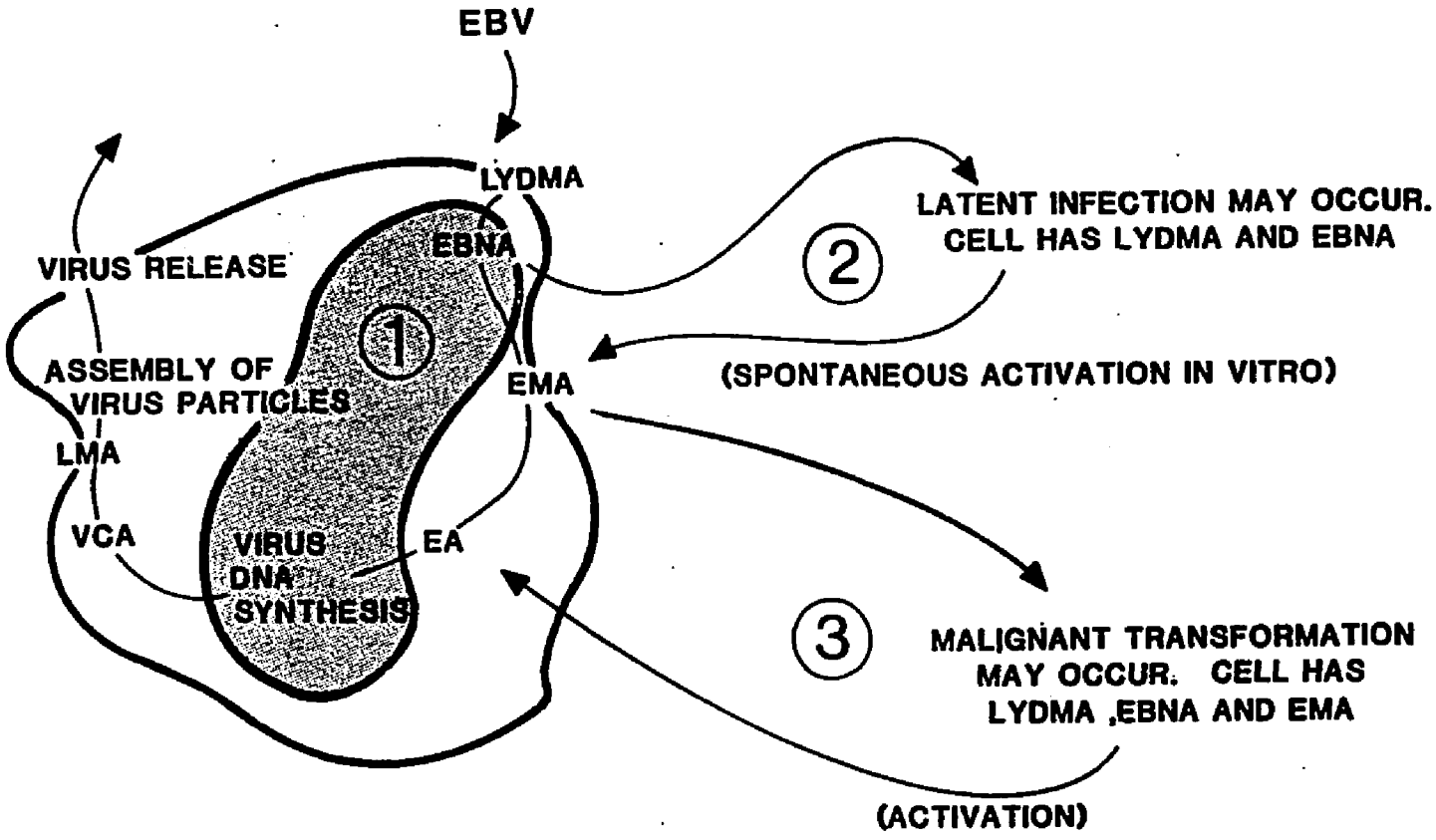

Figure 1. Diagram showing the expression of viral antigens in cells during various forms of infection by EBV(II). EBNA-BEV nuclear antigen, LYDM- Lymphocyte detected membrane antigen, EMA-Early membrane antigen, LMA - Late membrane antigen, EA - Early antigen, and VCA - Viral capsid antigen.

Burkitt's Lymphoma (BL) and nasopharyngeal carcinoma (NPC), the relationship of the virus to tumor cells closely resembles the type of infection seen with known oncogenic animcal DNA viruses.

EBV-Associated Nuclear Antigen (EBNA)

A number of new viral proteins must be synthesized before cellular transformation can begin. It has been established that 20 new proteins may be synthesized in the virus transformed cell (15), however, EBNA is the only protein that has been identified positively. EBNA is a DNA binding protein found in the nucleus of a transformed cell about 12-18 hours after the addition of the virus (16). Twenty-four hours later, the cell undergoes a morphologic change into a blast cell. Thirty-six hours later, the initiation of DNA synthesis can be detected by radio-autography, and cell proliferation begins.

The function of EBNA is not known. It is the only consistently exposed viral "footprint" in cells that carry EBV DNA, and it can be detected by a three layer anti-complement immune fluorescence technique (ACIF) (7).

Lymphocyte-Detected Membrane Antigen (LYDMA)

All EBV-transformed cells appear to express LYDMA, which is recognized by specific cytotoxic T-cells present during the acute phase of IM (17). So far the target

antigen has not been identified serologically, but it is different from the membrane antigen which is detected by fluorescence antibody tests. Cytotoxic T-cells with anti-LYDMA activity appear during the acute phase of IM in parallel with the large blast cells and disappear during convalescence. It has been suggested that LYDMA may occur before EBNA. This suggestion is based on the claim that during acute IM the EBV-infected LYDMA positive cells are EBNA negative (18).

Membrane Antigen (MA)

When the virus infects a cell, the viral envelope remains on the surface of the cell and thus a newly infected cell expresses membrane antigen. This expression is the early MA and is dependent on DNA synthesis. After DNA synthesis an additional membrane antigen occurs and is designated Late Membrane Antigen. Late Membrane Antigen can be differentiated from EMA by absorption and blocking tests (19).

Pearson and coworkers found a good correlation between EBV-neutralizing and anti-MA titers. These data suggest that anti-MA antibodies have neutralizing activity. Antibodies against cell surface antigens are also demonstrated by the antibody dependent cell mediated cytotoxicity (ADCC) test (20).

Early Antigen Complex (EA)

EA is subdivided into two components which differ in their serological specificity and their location within the cell. One type designated restricted (R) is found in the cytoplasm. The other type called diffused (D) is found in the cytoplasm and the nuclei. D appears before R during the early stage of the viral cycle.

Anti-EA sera from active IM patients contain Anti-D antibody more frequently than the Anti-R. The appearance of EA is the first definite sign that the cell has entered the lytic cycle, as double fluorescence techniques have shown all viral capsid antigen positive cells contain EA as well. Production of the EA-complex is not dependent upon DNA synthesis and in contrast to MA and VCA, EA cannot be demonstrated in the virion. It is therefore classified as a nonstructural antigen complex.

The Viral Capsid Antigen (VCA)

VCA was the first EBV antigen to be detected by indirect immunofluorescence techniques. The frequency of VCA-positive cells correspond to the number of cells containing virus particles. Anti-VCA antibodies react only with the naked virus, not with the envelope virus particle, suggesting that the VCA is a structural component of the virus capsid.

The receptor for EBV is present on peripheral B-lymphocytes (21). A variety of experiments has suggested

that the receptor is either identical to or closely associated with the receptor for C'3 (22). Evidence for this is obtained from experiments showing complete overlap and cocapping of the two receptors by immunofluorescence techniques and the blocking of C'3 receptors prevents the binding of EBV (23).

Following recognition and absorption to the cell membrane, the virus enters the cell. Penetration of the cell membrane may occur by pinocytosis or fusion, however, electron microscope observations suggest that fusion is the mechanism (24). The time for penetration of the virus is less than one hour, as measured by loss of ability of anti-EBV serum to inhibit transformation. During or immediately after penetration, the virus is uncoated and some "membrane antigens" representing the viral envelope are left at the cell surface. After uncoating a number of phenomenon may occur.

EBNA is expressed by 12 hours (25). After EBNA expression the virus DNA is replicated to give up to 60 genome equivalent per cell; following this genome amplification, some viral DNA molecules are linearly integrated into host cell DNA on one or more of chromosomes 3, 4, 5, 6, and 10 (26), while the remaining viral DNA remains free as circular molecules. Cellular transformation takes place at this time, and it has been suggested that it appears to be related to amplification of the genome load

or to the integration of some EBV DNA molecules (27). In any case, it should be noted that the transformation events and EBNA induction are two independent steps and that the whole viral genome appears to be necessary for transformation, but only about 5% of the genome is required to maintain the transformed state (28).

B-lymphocytes from seronegative subjects, when exposed to EBV, can be established as transformed cell linces. Seropositive individuals have in their peripheral blood a few latently infected cells that are activated to a productive infection when placed in cell culture. It is the infectious virus liberated in this way which infects new B-cells and transforms them into cell lines (29) in a process identical to that which occurs when the virus in added in vitro to B-lymphocytes from seronegative sources. Cell lines are also established when cells or explants of Burkitt's lymphoma are placed into cell culture. This is accomplished by the direct outgrowth of the malignant tumor cells.

EBV-carrying lymphoblastoid lines have been established from the lymphocytes of a variety of subhuman primates (marmosets, owl monkeys, gibbons, squirrels, and cebus monkeys) either by transformation with EBV in vitro or by culture of blood from the infected animal (30).

Immunity Against EBV, Humoral and Cell Mediated Immunity

Humoral responses are the responses evoked by lytically and nonlytically infected B-lymphoid cells with EBV-determined membrane antigens. The serologic response to the primary infection of EBV is well known. A transient evaluation of IgM titer to viral capsid antigen occurs in the first weeks of the illness and then gives way to anti-VCA IgG antibodies which remains present for life. Heterophile antibodies and neutralizing antibodies tend to arise later. Anti-EBNA antibodies emerge after many weeks or even months in convalescence, after the specific T-cell killing of EBNA containing B-cells. Anti-EA appear initially following the infection and is also transient in acute primary infection. Anti-EA and heterophile antibodies do not persist beyond six months, however, anti-VCA, and anti-EBNA and neutralizing antibodies may persist for years, if not for life (31). The profile of EBV-infected cell is shown in Figure 2.

The immune response includes the emergence of activated T-cells specifically primed against EBV-transformed lymphoblastoid cells and possibly also antibody dependent cellular cytotoxicity (20).

One model to describe the cell mediated immunity (CMI) is from the studies of Thorley, Lawson, Chess, and Strominger (33) in which adult lymphocytes (but not fetal)

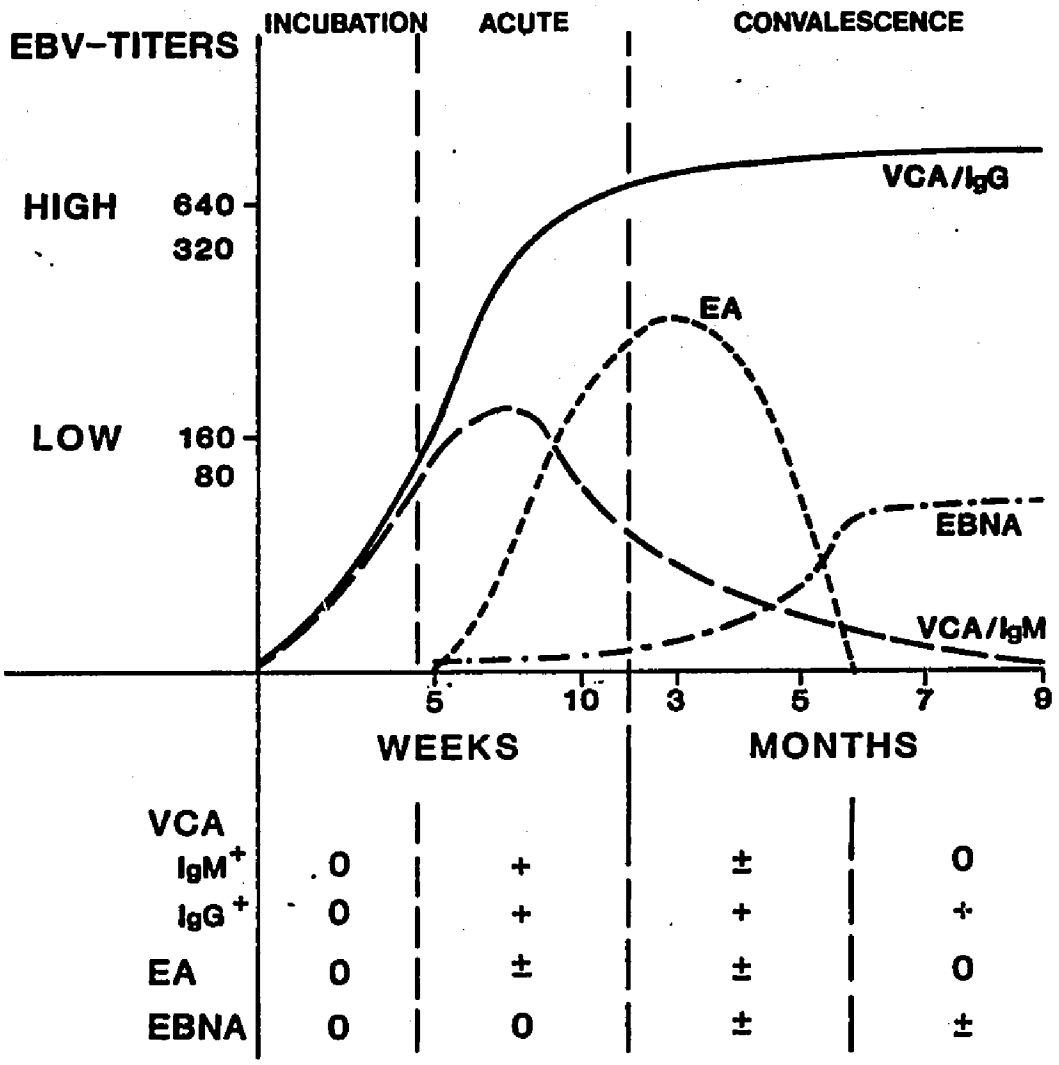

Figure 2. Comparison of antibody titers against viral antigens during infectious mononucleosis (32).

were shown to be capable of suppressing DNA synthesis and immortalization (establishment of lymphoblastoid cell lines) of EBV infected cells. This suppressive effect is not observed if T-cells are added to the infected B-lymphocytes later than 24 hours.

Other studies have described the activation of suppressor T-cell during acute IM, by demonstrating that normal B-cells stimulated with pokeweed mitogen failed to synthesize immunoglobulin (34). This activity does not involve cytotoxicity.

In normal, healthy adults, response to EBV infection includes the following humoral and cell mediated responses:

1. Neutralizing antibodies that are capable of blocking virus before infection of a cell.

2. T-cells that are capable of suppressing infected B-cell DNA synthesis, EBNA induction, transformation, and immunoglobulin synthesis.

3. T-cell specifically primed against EBV-transformed lymphoblastoid cells.

4. Killer T-cells (ADCC).

5. Natural Killer cells.

Medical Problems Created by EBV

Infectious Mononucleosis (IM). IM is a self-limited disease that has a peak incidence between 17 and 25 years of age, but can occur in children and older

adults. The incubation period is 5-7 weeks in young adults and possibly shorter in children.

The clinical illness often occurs after 4-6 days of headaches, chills and lassitude. The full blown mononucleosis syndrome persists for about 8 weeks and consists of fever, phargyngitis, lymphadenopathy, splenomegaly, periorbital edema, skin rashes, and mild hepatocellular dysfunction. Complications involve the development of autoantibodies, hemolytic anemia, and aggammaglobulinemia. Blood smears obtained during the 1st and 2nd week of illness characteristically consist of 60-80% lymphocytes and monocytes with 20-25% of these cells being classified as atypical lymphocytes. These abnormalities persist from 2 weeks to several months. The atypical lymphocytes have T-cell characteristics (34), however, other studies show that B-cells also contribute to the atypical lymphocyte pool (36).

In spite of the widespread lymphoproliferation and severe symptoms associated with IM, the disease is usually a benign, self-limited disorder. During the past decade, a growing number of patients with lethal infectious mononucleosis have been described (37). The work of Purtilo and others has established that in a number of families, the affected individual suffers from X-linked lymphoproliferative syndrome (XLP). Study of this disease has

contributed much in understanding the host defense against EBV infection.

Fatal mononucleosis is characterized by depletion of T-cells in lymph nodes and spleen, plasmacytosis in hematopoietic organs, infiltration of the brain and viscera by lymphocytes and plasma cells, and deficient anti-EBV antibody production (38).

The X chromosome appears to govern many immune functions. The XLP presumably reflects a defect at a locus of the X-chromosome which is important for control of lymphoproliferation.

Burkitt's Lymphoma (BL)

BL was named after Denis Burkitt, who recognized the lymphoid tumors of children in Africa. Its contribution is across tropical Africa, and the territories of Paua and New Guinea (39). Virus particles recognized as members of the herpes virus were observed by electron microscopy of a continuously growing BL-derived lymphoblast culture (40), but the evidence implicating that EBV was the causative agent in BL was derived from serologic, epidemiologic, molecular, and biological studies. Lymphoblastoid cell lines derived from BL differ in their chromosomal constitution from cell lines derived from IM patients. In addition to the aforementioned data, BL lines exhibit an 8:14 chromosomal translocation (41).

The 8:14 translocation has been described in American Burkitt's lymphoma, and no EBV containing tumor has ever been described without it (42). The presence of chromosome 14 translocation in some EBV-negative lymphomas suggests that this chromosomal locus is important for the evolution of a lymphoma.

George Klein has proposed a 3-step hypothesis to explain EBV induced lymphomagenesis. He suggested that the initial step in African Burkitt's lymphoma was the EBV-induced immortalization of some B-lymphocytes after primary infection (42). The second step was an environment dependent factor, such as holoendemic malaria. Malaria is immunosuppressive. The third and final step was the development of Burkitt's lymphoma in the occurrence of the 8:14 translocation (43). The 8:14 translocation is also found in EBV negative American BL (43).

Naso-Pharyngeal Carcinoma (NPC)

The first association between EBV and NCP was made following the discovery of EBV-related antigens in the sera of American NPC patients (44). Additional immunoflourescence and DNA-DNA hypridization studies confirmed the relationship (45).

The association of EBV with the carcinoma in NPC raises the question of the mode of infection. Do epithelial cells have EBV receptors or are they infected

by transfer of viral information from lymphoid cells? As proposed by Huesen (1975), the transformation of the epithelial cells takes place within the vicinity of permissive (EBV-synthesizing) cells. Fusion of EBV-carrying cell and epithelial cells may be the mode of entry of the virus to epithelial cell devoid of virus receptors. Lymphoblastoid cells, in non-lytic cycle containing thousands of copies of the viral genome could possible fuse and transfer the entire viral genome load to the epithelial cells load (46).

Chronic EBV

Chronic EBV is perhaps a disease condition of long standing and only in the past few years has it been recognized by the clinician and researcher. It appears that chronic EBV manifests itself in a small percentage of people who recover from infectious mononucleosis. The symptoms show considerable variation from one patient to another but the most noted are fatigue, pharyngitis, dizziness, malaise, paresthesias, paresis, changes in emotional status, blurring of vision, sleep disorders, myalgia, and depression.

Discussion with many patients having chronic EBV has revealed that many patients have hypersensitivities towards environmental agents. Based upon this observation,

we believe that a positive correlation exists between the presence of hypersensitivity disorders and the inability of the body to manage properly the EBV.

This is a preliminary study to detect the sensitivity of peripheral blood lymphocytes (PBL) from these groups of people towards common allergens. The three groups of people are: EBV-positive mild hypersensitive people, EBV-positive hypersensitive healthy people and EBV-positive hypersensitive ill people (chronic EBV). A correlation between the allergen, lymphocyte stimulation response, clinical signs, and EBV serology will be made. Then in each case, PBL will be placed into cell cultures with the culprit allergen, and then the cultures will be assayed for EBNA-positive cells to determine if the cells from the chronic EBV group show greater numbers of EBNA-positive cells than the other two groups.

CHAPTER 2

MATERIALS AND METHODS

Materials

Peripheral Blood Lymphocytes (PBL) and Serum Samples

Blood was obtained at two different times from three groups of people: EBV-positive mild hypersensitive subjects, EBV-positive hypersensitive healthy subjects, and EBV-positive hypersensitive ill subjects (chronic EBV). In each case the blood was collected via intravenous puncture, and immediately defibrinated by shaking in plastic cubes with 5 mm diameter acid washed glass beads. The blood samples were separated to obtain serum and PBL samples as described later in the basic procedure.

Allergens

Allergens representing common pollens and other environmental reagents were purchased from Hollister-Steir (Division of Cutter Laboratories Inc., Woodland Hills, California). Each allergen preparation was dialyzed using size 8 dialyzing tubing, against four liters of saline for 72 hours. The saline was changed four times in the 72 hour period. Allergens were sterilized by passing them through a 0.45 um diameter Millipore filter, portioned

into 1 ml quantities and stored at 4°C. Allergen obtained from Hollister-Steir included: Bermuda grass, Johnson grass, privet, olive, mulberry, mesquite, ragweed, dog dander, and cat dander. Herpes Simplex Virus One and EBV antigens, gifts from Dr. R. Pritchett, The University of Arizona, and Coccidiodes immitus antigen (1:3 dilution, Travenol Laboratories, Costa Mesa, California) were portioned into 0.2 ml quantities in small vials and stored at -20°C.

Culture Medium

RPMI 1640 (Gibco, Grand Island, New York) was reconstituted according to directions. The RPMI 1640 culture medium contained Hepes buffer at a concentration of 0.0825 mg/ml, and L-glutamine (Nutritional Biochemicals Corp., Cleveland, Ohio) at a concentration of 2 mM. The medium was adjusted to a pH of 7.2 and 7.4 and then supplemented prior to use with complement inactivated Fetal Calf Serum (FCS, Gibco Laboratories) cona concentration of 20%.

AET-Treated Sheep Erythrocytes (SRBC)

Sheep blood was obtained from a designated ewe housed at The University of Arizona Experimental Frams. Blood was collected in Alsever's solution (Appendix A) every two weeks.

SRBC preparations to be used for PBL rosetting procedures were treated with AET (2 aminoethylisothiouronium bromid hydrobromide, Sigma Chemical Co., St. Louis, Missouri) to increase the stability of the cell membranes (47). SRBC in Alsever's solution were washed four times in phosphate buffered saline (PBS, pH of 7.2) and then centrifuged at 400 x g for 10 minutes to yield a packed cell volume. 1.96g at AET was dissolved in 35 ml of distilled water and the pH was adjusted to eight. Four volumes of AET were added to one volume of washed packed SRBC, incubated at 37°C for 25 minutes with intermittent vigorous shaking and then washed three times with PBS. The treated cells were stored at 4°C and used within a 48 hour period.

SRBC Absorbed FCS

SRBC used in PBL rosetting procedures was treated as follows. One volume of packed washed SRBC was mixed with ten volumes of heat inactivated (56°C for 30 minutes) FCS, incubated at 37°C for 30 minutes and then incubated a second time at 4°C for 30 minutes (47). The preparation was centrifuged at 400xg for ten minutes and the supernatant fluid was then sterilized by passing it through a 0.45 um Millipore filter. The finished product was portioned into small vials and stored at -20°C.

Poly L-Lysine Treated Slides

Microscope slides, for the assay of EBNA, were washed with a soap solution, rinsed with distilled water and then immersed in poly L-lysine (50 mg/liter, Sigma Chemical Co., St. Louis, Missouri) for 45 minutes at room temperature. Slides were rinsed in several changes of PBS and stored at 4°C (48).

Basic Procedures

Preparation of Serum Samples

Seven to ten mls of defibrinated blood from each subject was placed into a sterile tube and centrifuged at 2500 rpm for 10 minutes. The serum was removed, divided into two portions for EBV serology and stored at -20°C.

Preparation of PBL Samples

The defibrinated blood from each subject was diluted threefold with PBS, layered over one volume of Ficoll-Hypaque fluid (Appendix A) and centrifuged at 200 xg for 30 minutes (49). The mononuclear cells were removed from the interface and washed three times with Eagle's minimum essential medium (MEM, Appendix A). The viability and total numbers of PBL for each preparation was determined by regular cell counting procedures using trypan blue as the indicator dye. The PBL preparations were used directly in the lymphocyte stimulation test or purified further to obtain pure B-cells.

Rosette Procedure to Obtain B-Lymphocytes

T lymphocytes can be removed from PBL by rosetting the cells with SRBC (47). PBL were diluted to $1x10^7$ cells per ml in Eagle's MEM in a 50 ml round bottom siliconized tube. A final 1% (vol/vol) mixture of AET treated SRBC was made by adding the previously treated SRBC.

The mixture was incubated at 37°C for 10 minutes, centrifuged at 200 xg for 10 minutes, then placed upright in an ice bath for 60 minutes. All but 7.5 ml of the supernatant fluid was removed without disturbing the cell pellet. The tube was then placed in a horizontal position and rotated gently to resuspend the cell pellet, which was then layered over a Ficoll-Hypaque gradient and centrifuged at 300 xg for 40 minutes at 23°C. The rosetted T cells are removed leaving the purified B cells at the interface. The B cells were removed, washed three times with Eagle's MEM and suspended at a concentration of $1x10^5$/ml in culture medium.

Purity of the separation was checked by an indirect immunofluorescence technique. $5x10^5$ cells were mixed with 5ul of mouse antiHuman Leu 1 (Becton Dickinson C., Mountain View, California) for 20 minutes at 4°C, washed three times with PBS, and then incubated with 1 ul of fluoresceinated Goat immunoglobulin (Becton Dickinson Co.) for 20 minutes at 4°C. The final cell suspension was

washed three times with PBS and the percentage of labeled T-cells was determined.

Procedure For Preparation of EBV

Virus preparations were grown on a transformed B lymphocytic line (B_{95-8}) maintained in the laboratory (50). Supernatant fluid was removed 12-13 days after the cultures had been split, and centrifuged at 300 xg for 10 minutes at 4°C to remove cellular debris. The supernant fluid was centrifuged a second time over a sucrose gradient (1 ml of 40% sucrose) at 30,000 xg for 2.5 hours at a maximum temperature of 10°C. The pellet was suspended in 1 ml MEM and assayed for virus activity using the lymphocyte stimulation assay. A 1 to 10 dilution usually proved to be most effective. The purified virus was placed in small amounts in vials and stored at -70°C.

Lymphocyte/Stimulation Assay

PBLs from each subject were separated from whole blood as described earlier. The cells were diluted to $1x10^6$ cells per ml of RPMI 1640 culture medium, and 0.1 ml of the cell suspension was placed in each well of a Limbro Microtiter place (51). Cells were cultured with at least 10 different allergens. Each allergen was checked in triplicate at three concentrations (10 ul, 20 ul, and 30 ul per well). A final volume of 0.2 ml was maintained in each well. Cultures were incubated at 37°C for five

days in a humidified 5% CO_2 chamber and then pulsed with 1 uCi of tritiated thymidine (specific activity 5 Ci/mMol, Amersham Searle Corp.) for 24 hours.

The cultures were harvested onto glass fiber filters using an automated instrument (mini-Mash, Bio-products, Walkersville, Maryland). The filters were air dried overnight at room temperature, then placed into scintillation fluid (PRO-POPOP toluene, Appendix A), and the amount of radioactivity was determined on a Packard TriCarb Liquid Scintillation Counter (Packard Instrument Co., LaGrange, Illinois, Model 332). Sample vials were counted for one minute at a counting efficiency of 37.5%.

Data were expressed as CPM for each sample, and mean values for each set of triplicates were determined. Stimulation Indices were calculated as:

$$\frac{\text{mean cpm of 3 samples + allergen}}{\text{mean cpm of 3 samples without allergen}}$$

A Stimulation Index of 2.0 or greater indicates the lymphocytes of the subject responded positively to that particular allergen.

Establishment of EBV Transformed Cell Lines

B-cells from each subject were placed into cell culture at a concentration of $5x10^5$ cells/ml. Medium consisted of RPMI 1640 + 10% PCS, gentamycin and buffer as described above. Two cultures were made from the B-cells of each subject. One of the cultures was infected with

100 ul of stock EBV per $1x10^6$ cells (52); the other cell culture was left as is to determine if transformation would occur. Both cultures were kept at 37°C in 5% CO_2 atmosphere and all cultures were observed periodically for evidence of transformation. The appearance of floating clumps of cells after 8-30 days was evidence for transformation and establishment of the cell line. After a minimum of 30 days, the "established" cells were removed, centrifuged and resuspended at $1x10^6$/ml in culture fluid containing 10% DMSO, then placed at -20°C for 24 hours and finally stored at -70°C.

Detection of Antibodies to EBV

Anti-VCA, anti-EA and anti-EBNA was determined from the serum of each subject. The assays were conducted by the Virology Clinical Laboratories of the Arizona Health Center and the procedures are recorded in Appendix A.

Detection of EBNA Positive Cells

Purified B-cells and mixtures of T- and B-cells from each subject were cultured fro up to 16 days in the presence of and absence of the culprit allergen. Two x 10^6 cells per ml of RPMI 1640 + 10% FCS, gentamycin and Hepes buffer were incubated at 37°C in 5% CO_2. Approximately $4x10^5$ cells were removed at 0, 1, 2, 3, 4, 8, and 16 days and the cells were assayed for EBNA by the

anticomplement immunofluorescence method (ACIF) of Reedman (53). At each time period, the cell suspension was washed three times in fresh PBS (pH 7.2) and then resuspended to make a slightly cloudy cell suspension. Ten ul containing approximately $1x10^4$ cells were placed upon a poly lysine treated microscope slide which was mounted in an IEC microscope slide carrier (IEC, Needhambts, Mass.). The preparation was centrifuged at 1100 rpm for five minutes, then the slide was allowed to air dry, fixed in acetone at -20°C for ten minutes and stored at 20°C until assayed.

The assay consisted of overlaying the cell preparation with a 1:8 dilution of heat activated EBNA positive human serum. The cells were incubated at 35°C for 60 minutes in a humid chamber, washed two times for five minutes each in PBS, overlayed with a 1:25 dilution of fresh guinea pig serum (Appendix A) at 35°C for 60 minutes in a humid chamber. Once again the slides were washed two times in PBS and then overlayed with a 1:80 dilution of Anti-C'3 fluorescein conjugate (Cappel Laboratories, Inc., WEst Center, PA). This preparation was washed as described earlier and the cell preparation was covered with a 10% glycerol in PBS mixture and observed with a phase contact fluorescence Ploem illuminated microscope (Lietz Wetzlar, Germany). The percentage of cells showing typical fluorescence per 200-400 cells were recorded. Controls

using complement, anticomplement, and serum anticomplement were done. Results were expressed as the percentage of EBNA positive cells per time period per subject.

Protocols

Protocol One

Serum and PBL from EBV-positive mild hypersensitive, EBV-positive hypersensitive healthy and EBV-positive hypersensitive ill people were obtained. The serum and cells from each subject was treated according to the following procedure: (1) the serum sample was assayed for Anti-EA, anti-VCA and anti-EBNA antibodies, (2) PBL were placed into cell culture with approximately ten different allergents to ascertain an individual's hypersensitive status, and (3) a portion of PBL were rosetted with SRBC to obtain a purified population of B-cells which were placed into cell culture with and without the addition of EBV. This was done to established transformed B-cell lines, of perhaps different idiotypic specificities, to be used in future work.

The data from each subject were analyzed individually and collectively to (1) ascertain the existence of the three desired groups, (2) to judge the similarities or differences in the incorporation of ^{3}TdR into DNA of the cells exposed to different types of allergens such as three pollens, grass pollens, Coccidiodes immitis, and

dander allergens, (4) to correlate the serological data, and (5) to correlate the serological data with the stimulation index data obtained from the testing of atopic responsiveness.

Protocol Two

This part of the work was to determine the existence and emergence of EBNA positive B-cells in cell cultures of each subject following in vitro stimulation with the culprit allergen. PBLs from each subject were divided into two portions and treated as follows: One part was rosetted with SRBC to remove the T-cells and the B-cells were checked by immunofluorescence techniques to ascertain the purity. The purified B-cell preparation and the original T- and B-cell population were diluted to $2x10^6$ cells per ml to RPMI 1640 culture medium + 10% FCS and placed into culture with 100 ul of the culprit allergen, 100 ul tetanus toxoid or 100 ul of culture medium as a control.

Samples of cells from each preparation were obtained at 0 time and 1, 2, 4, 8, and 16 days later and analyzed for the presence of EBNA positive cells. Three hundred cells were counted for each sample.

The data from each subject were analyzed individually and collectively to (1) determine the original number of EBNA positive cells per subject and the changes

caused by the culprit allergen and the control antigen, tetanus toxoid, (2) to correlate the results with the previous stimulation index data, and (4) to correlate all data to the existence of the three groups of experimental subjects.

CHAPTER 3

RESULTS

The original goal of this study was to determine the EBV serology and hypersensitivity status of enough people to obtain at least ten subjects in each of three groups; chronic EBV, EBV-positive hypersensitive people and EBV-positive mild hypersensitive people.

In total, serum and PBL from 51 subjects were analyzed. Six were found to have negative EBNA serology and are not included in this study. Sufficient lymphocyte stimulation data was obtained on 40 of the 45 remaining EBNA positive subjects. Inspection of individual responsiveness to such allergens as olive, bermuda, ragweed, mulberry, cocci, mesquite, dog, cat, and house dust showed that any given individual responded with a stimulation index of two or more to at least one of the allergens.

To overcome the inherent problems in this finding, we determined the number of allergens each individual responded to, and the grand stimulation index mean (SI of each allergen divided by number of allergens) of each individual. Individuals with grand SI means of less than 1.7 and who had responded to less than one-half of the

allergens were classified as EBV-positive mild hypersensitive subjects. Individuals with grand SI means greater than 1.7 and who had responded to more than one-half of the allergens were classified as EBV-positive hypersensitive subjects. Table 1 presents these data and shows that 38 of the 40 subjects would fit this classification scheme. Twelve are classified as chronic EBV subjects, 14 as EBV-positive hypersensitive subjects and 12 as EBV-positive mild hypersensitive subjects. The 12 chronic EBV subjects have positive responsitive of 50 to 100% towards the allergens with grand mean stimulation index range of 2.6 to 25.4.

Table 2 presents this problem of classification in a different perspective. The chronic and non-chronic EBV subjects are identified by identification numbers. Inspection of the identification numbers show that the non-chronic EBV subjects vary between being classified as hypersensitive subjects or non-hypersensitive subjects, depending upon their responsiveness towards a certain allergen. However, the group averages for the chronic EBV subjects for the six allergens shown in Table 2 indicate a nearly two to five fold greater response than the EBV-positive hypersensitive subjects.

Table 1. Comparison of stimulation indices and the number of allergens showing positive responses in three groups of EBNA-positive hypersensitive subjects.

Chronic EBV			EBNA Positive Hypersensitive Subjects			EBNA Positive Mild Hypersensitive Subjects		
ID	SI Grand	Allergens: Positive/ Total	ID	SI Grand	Allergens: Positive/ Total	ID	SI Mean	Allergens: Positive/ Total
1	25.2	7/7	9	4.2	4/4	12	1.0	1/4
2	25.4	7/7	15	3.1	6/8	19	1.3	1/7
3	8.5	7/7	16	5.1	3/7	25	1.7	2/5
4	7.6	5/5	17	2.2	3/7	31	1.5	2/7
8	7.1	4/6	22	3.1	4/4	36	1.5	2/7
13	3.2	6/7	26	2.8	2/5	38	1.0	0/6
18	4.4	6/8	38	5.5	4/7	44	1.6	3/7
21	5.4	8/8	32	1.8	4/7	45	1.3	1/7
27	6.7	7/7	32	4.2	5/7	46	1.3	2/7
30	2.6	6/8	34	2.8	6/7	47	1.6	2/6
35	4.0	4/6	39	1.9	2/4	47	1.6	2/6
42	3.1	5/6	40	2.0	5/7	49	1.7	3/6
			41	1.7	6/7	51	1.0	0/2
			43	2.7	6/7			

Table 2. Mean stimulation index responses to certain allergens in the chronic EBV, EBNA-positive hypersensitive and EBNA-positive non-hypersensitive subjects.

GROUP	BERMUDA	RAGWEED	COCCI	MULBERRY	OLIVE	MESQUITE
CHRONIC EBV	10.3 ± 4.0 1,2,3,4,18,21,27,30	9.3 ± 6.0 1,2,3,4,8,18,27,42	10.6 ± 3.4 1,2,3,4,13,18,21,27	27.2 ± 23 1,2,3,4,8,13,27,30, 35,42	3.8 ± 0.7 1,2,3,4,13,18,21,27	8.1 ± 6.0 1,2,3,13,18,30, 35,42
EBNA$^+$ HYPERSENSITIVE	2.2 ± 0.2 5,9,16,22,23,29	2.3 ± 0.3 15,16,29,43	4.8 ± 2.1 22,25,29,30,34,39, 43,44,46,47	5.3 ± 3.7 15,31,32,34,37,40, 41,43,44	2.0 ± 0.4 15,30,31,34,41,42, 45,49	2.1 ± 0.1 32,34,37,41,43,45
EBNA$^+$ NON HYPERSENSITIVE	1.1 ± 0.2 12,15,19,25,28, 31,32	0.7 ± 0.4 37,39,40,41,44, 45,46,47,49	1.4 ±0.3 26,31,35,36,38,41, 42,45,48,49,51	0.8 ± 0.7 19,29,36,48	1.2 ± 0.3 12,25,29,35,36,43, 44,46,48	1.1 ± 0.5 13,22,29,31,36,38, 40,44,47,48

Serology for each of the 40 subjects is shown in Figure 3. Each subject had an anti-EBNA titer greater than 2. The bivariate mean of the anti-VCA (IgG) and anti-EA titers for each subject shows a separation into two groups. Subjects with complaints such as fatigue, pharyngitis, dizziness, malaise, paresthesias, paresis, blurring of vision, sleep disorders, and depression have anti-VCA titers greater than 160 and anti-EA titers greater than 80. The bivariate mean for the non-chronic EBV subjects is located in the lower left portion of the Figure.

An important difference between chronic EBV and non-chronic EBV was observed when the degree of ^{3}HTdR incorporated into the DNA of non-allergen stimulated PBL was compared. Figure 4 shows a mean value of 2943+1621 cpm of isotopse incorporated into cells of chronic EBV subjects compared to 624+421 and 554+404 cpm incorporated into the cells of the two groups of non-chronic subjects.

To ascertain the existence of three desired groups, specific responses to certain antigens were compared. Figures 5 and 6 show the stimulation indexes for nine subjects from group to olive, mesquite, mulberry, ragweed, bermuda, and cocci antigen. Chronic EBV subjects were shown to have higher responsiveness over the other two groups of subjects.

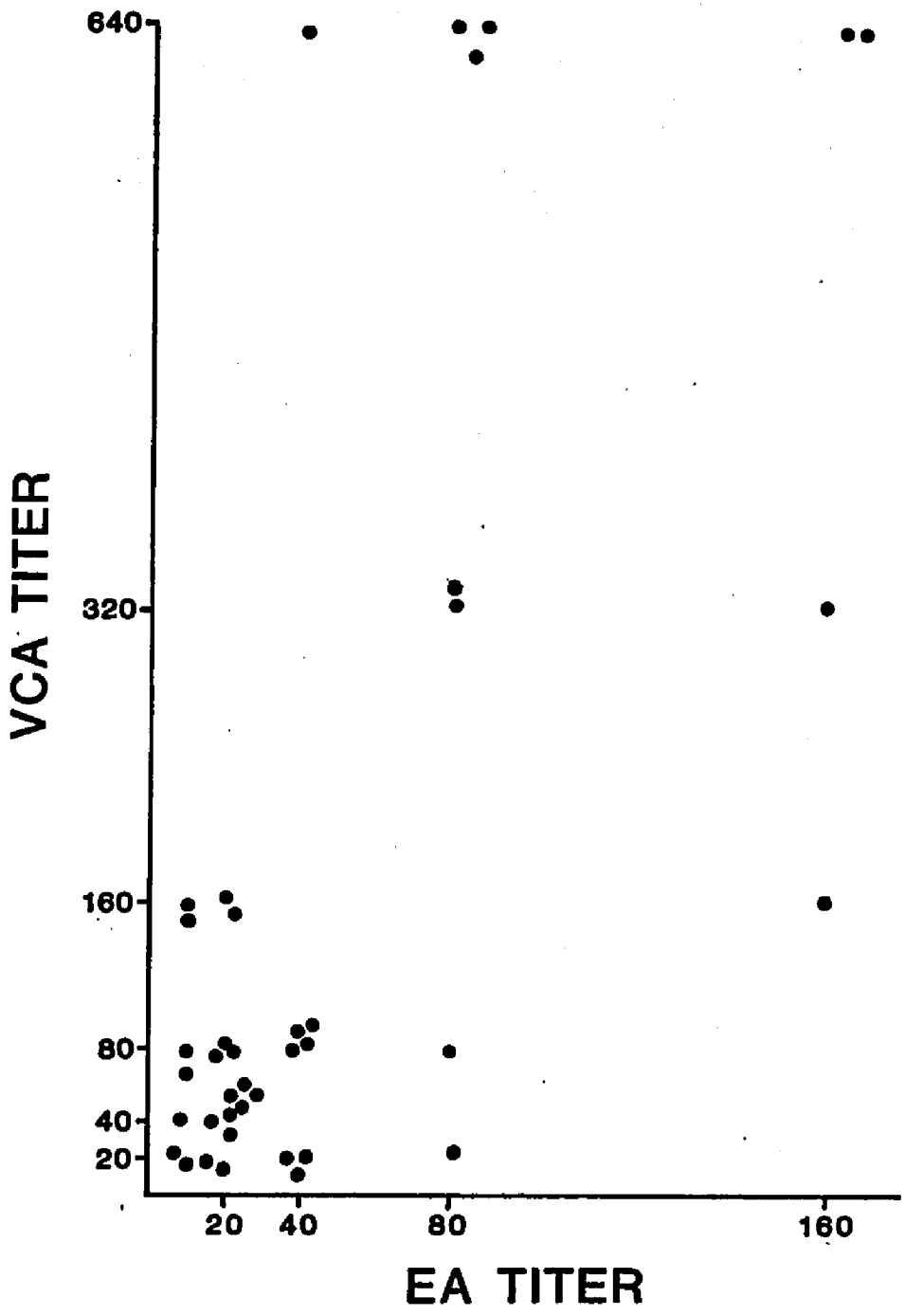

Figure 3. Bivariate analysis of EA-titers versus VCA-titers of subjects shown to be EBNA-positive.

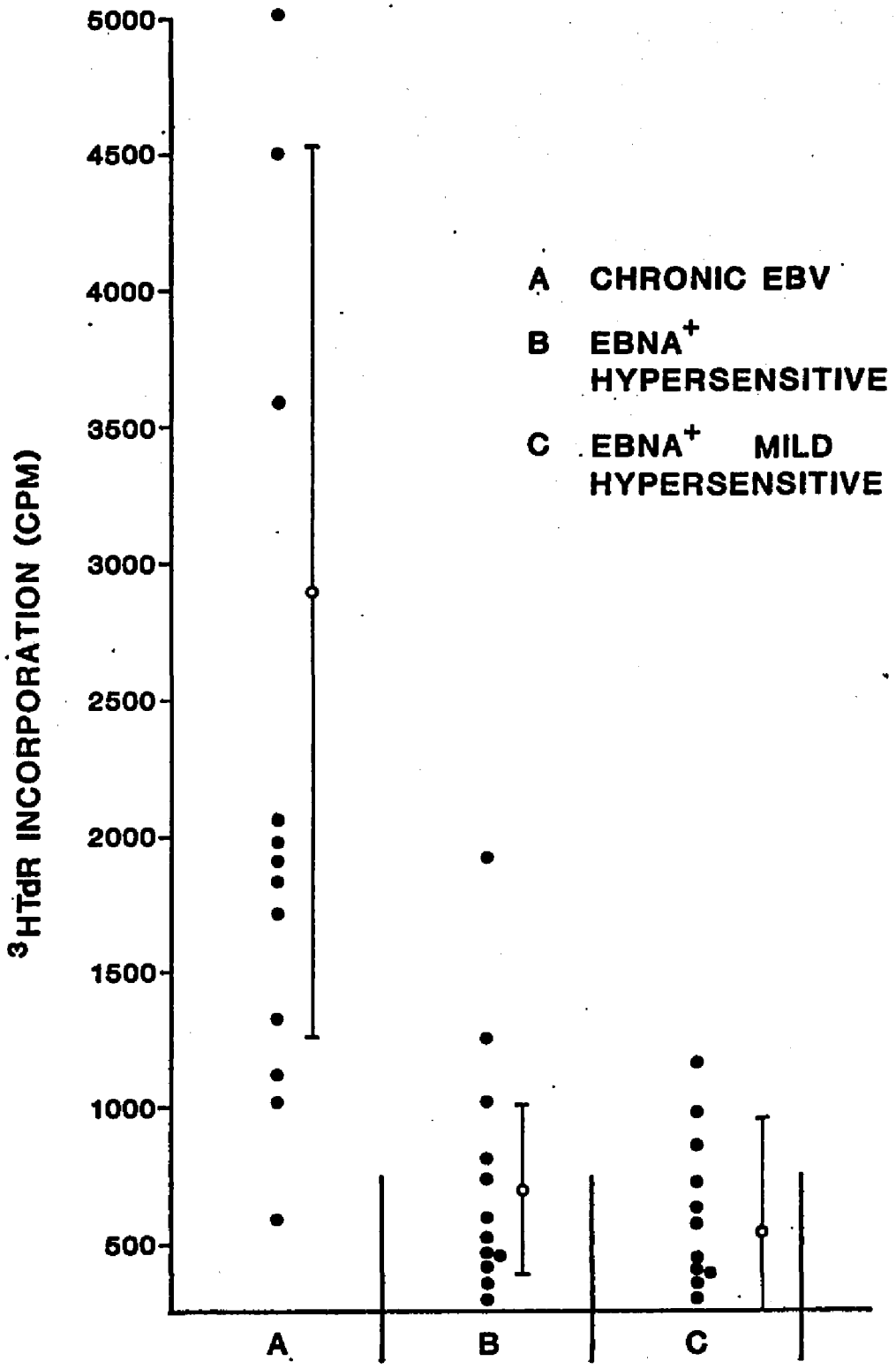

Figure 4. Incorporation of ^{3}HTdR into DNA of non-stimulated PBL in the three groups of EBNA-positive hypersensitive subjects.

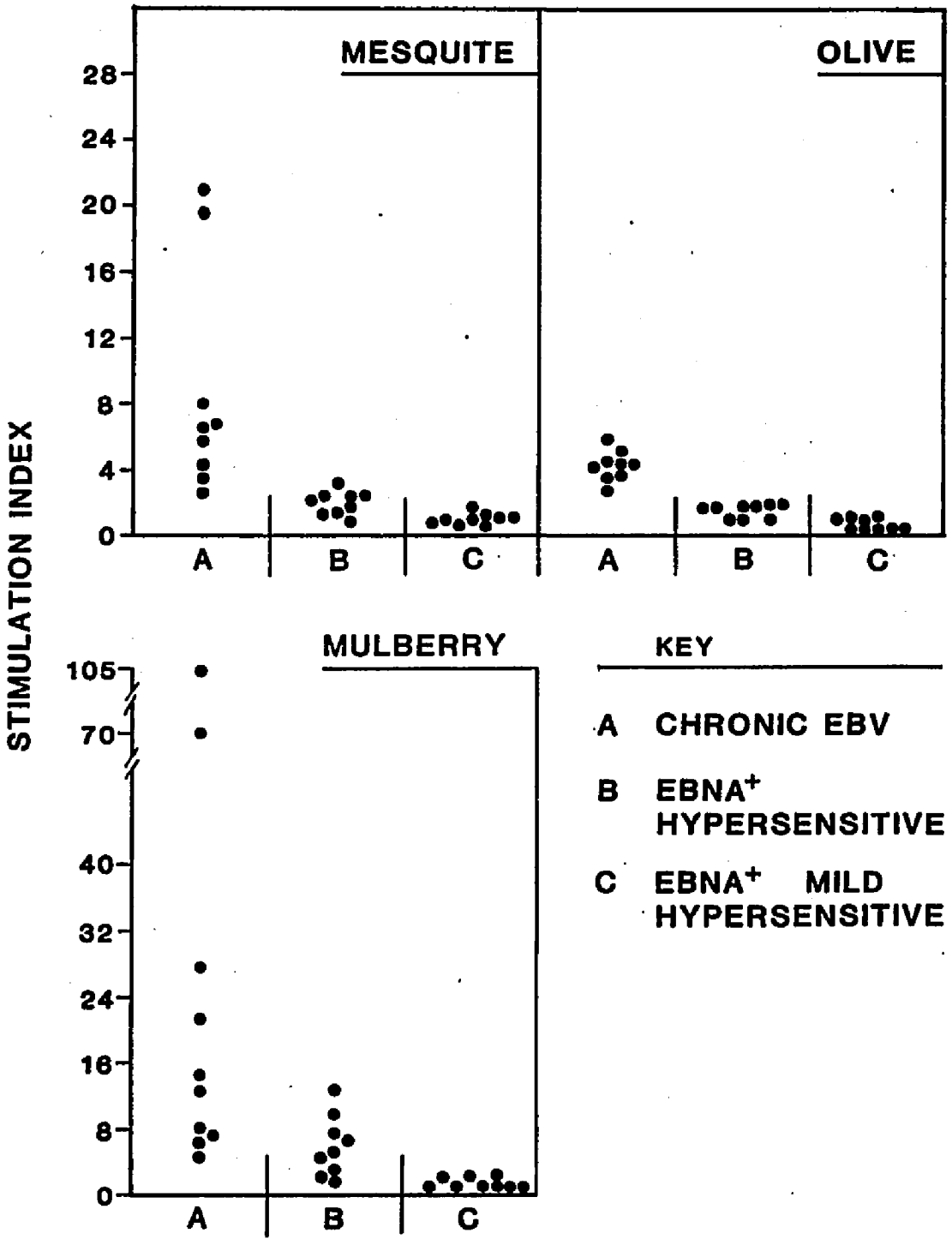

Figure 5. Comparison of responsiveness to mesquite, olive, and mulberry in the three groups of EBNA-positive hypersensitive subjects.

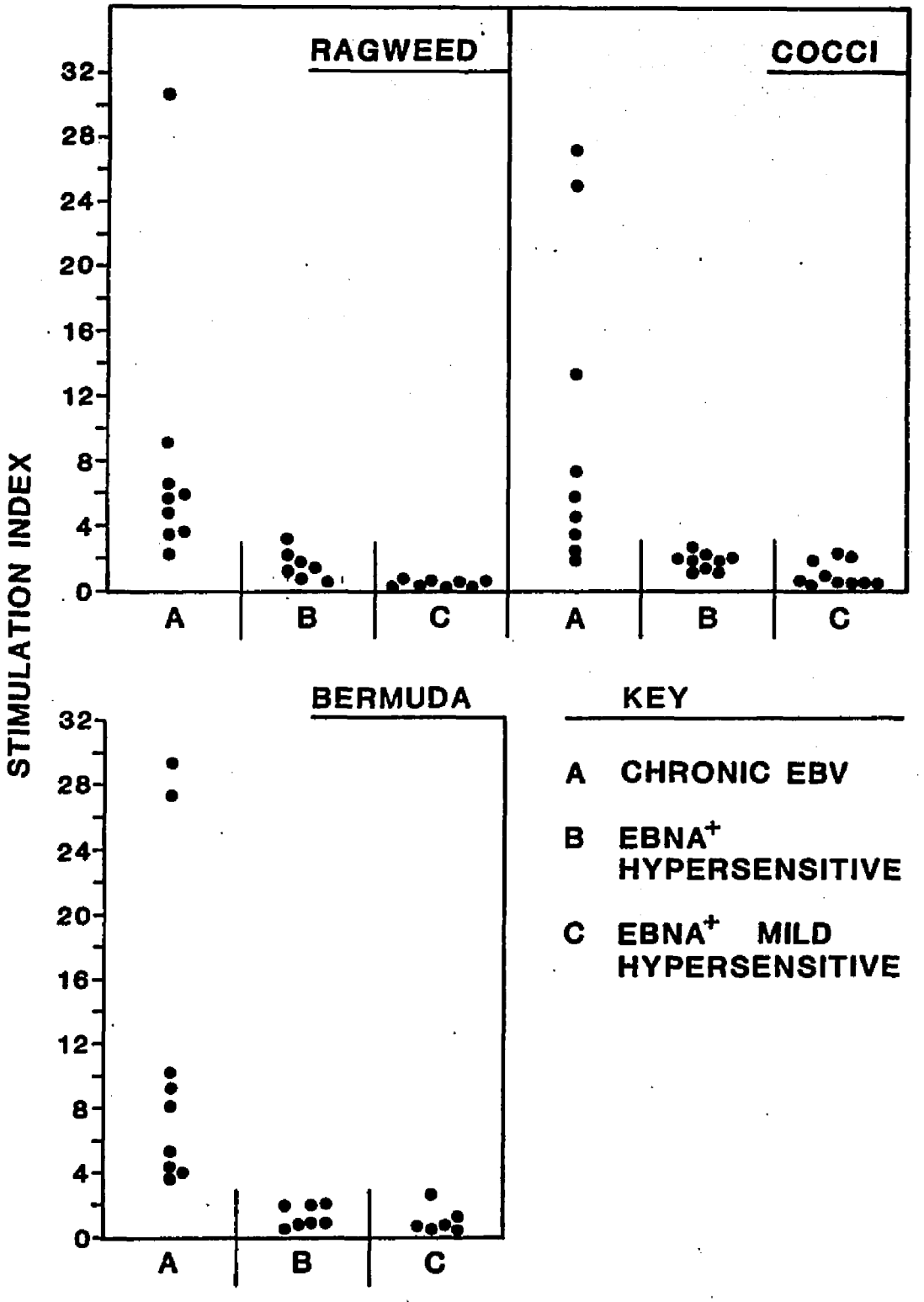

Figure 6. Comparison of responsiveness to ragweed, cocci and bermuda in the three groups of EBNA-positive hypersensitive subjects.

A second goal of this study was to determine the existence and emergence of EBNA-positive B-cells in cell cultures of the subjects in each group. B- and T-cells and purified B-cells from each subject were cultured alone, with tetanus toxoid antigen as a control and with the culprit antigen. The culprit antigens included the following: Bermuda, mulberry, cocci, HSV_1, olive, cat, and mesquite. Samples of cells from each preparation were obtained at 0 time and 1, 2, 4, 8, and 16 days later and analyzed for the presence of EBNA positive cells. Figures 7, 8 and 9 show the percentage of EBNA positive cells in the B-cell cultures from the chronic EBV, EBNA-positive hypersensitive and EBNA-positive mild hypersensitive subjects, respectively, while Figures 10, 11 and 12 depict the results observed in B- and T-cell cultures. Chronic EBV subjects in both B-cell and B- and T-cell cultures demonstrated a greater incidence of EBNA-positive cells at zero time and a greater increase in EBNA expression as a function of time when compared to the other two groups. Three subjects in the chronic EBV group showed less of a response than the other six subjects and this observation is quite apparent in the T- and B-cell cultures. Furthermore, the presistence of EBNA-positive cells in the two non-chronic groups was shown to be only in the B-cell cultures.

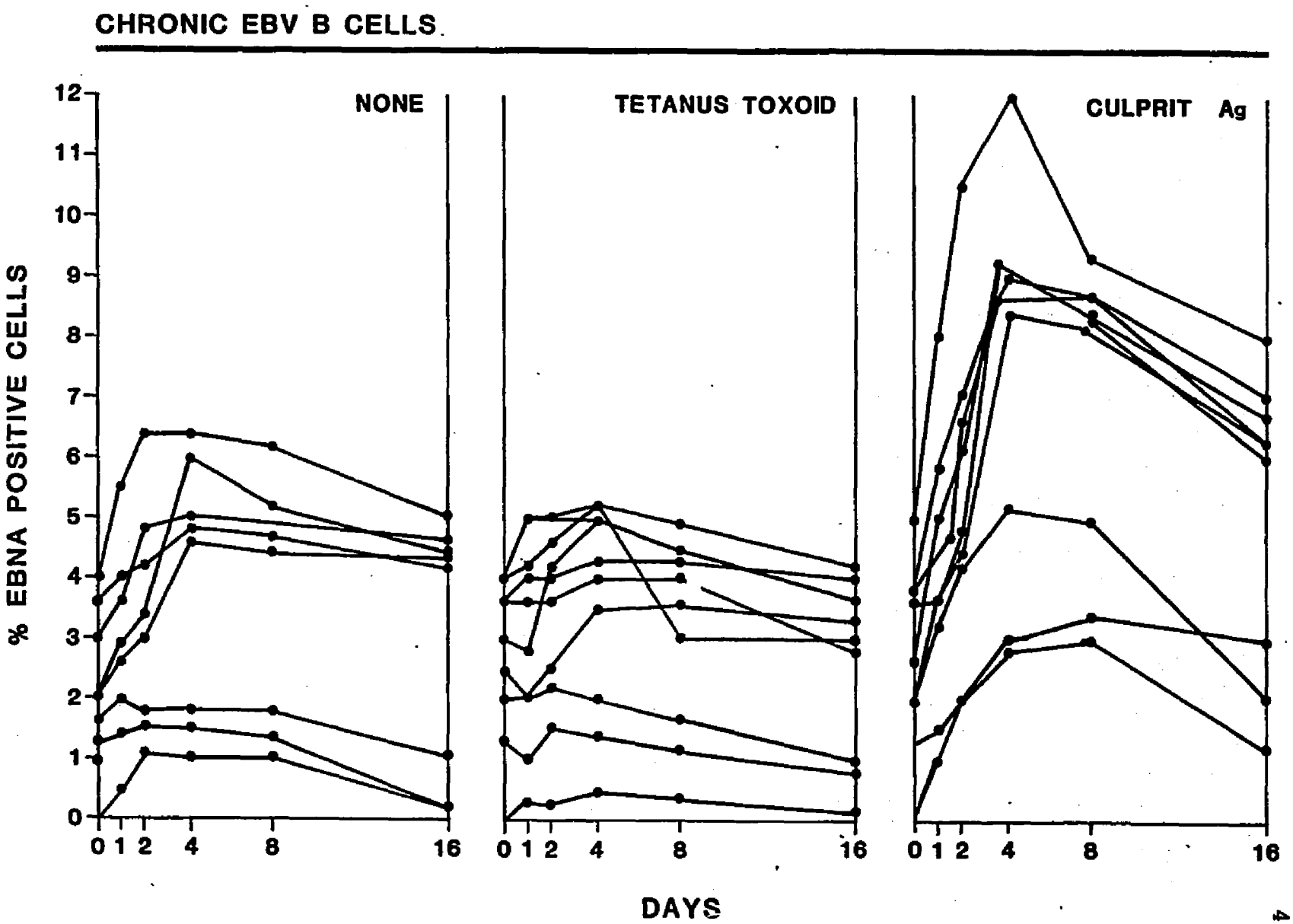

Figure 7. Percent EBNA-positive cells as a function of time in the B-cells cultures from chronic EBV subjects when incubated alone, with tetanus toxoid and with the culprit antigen.

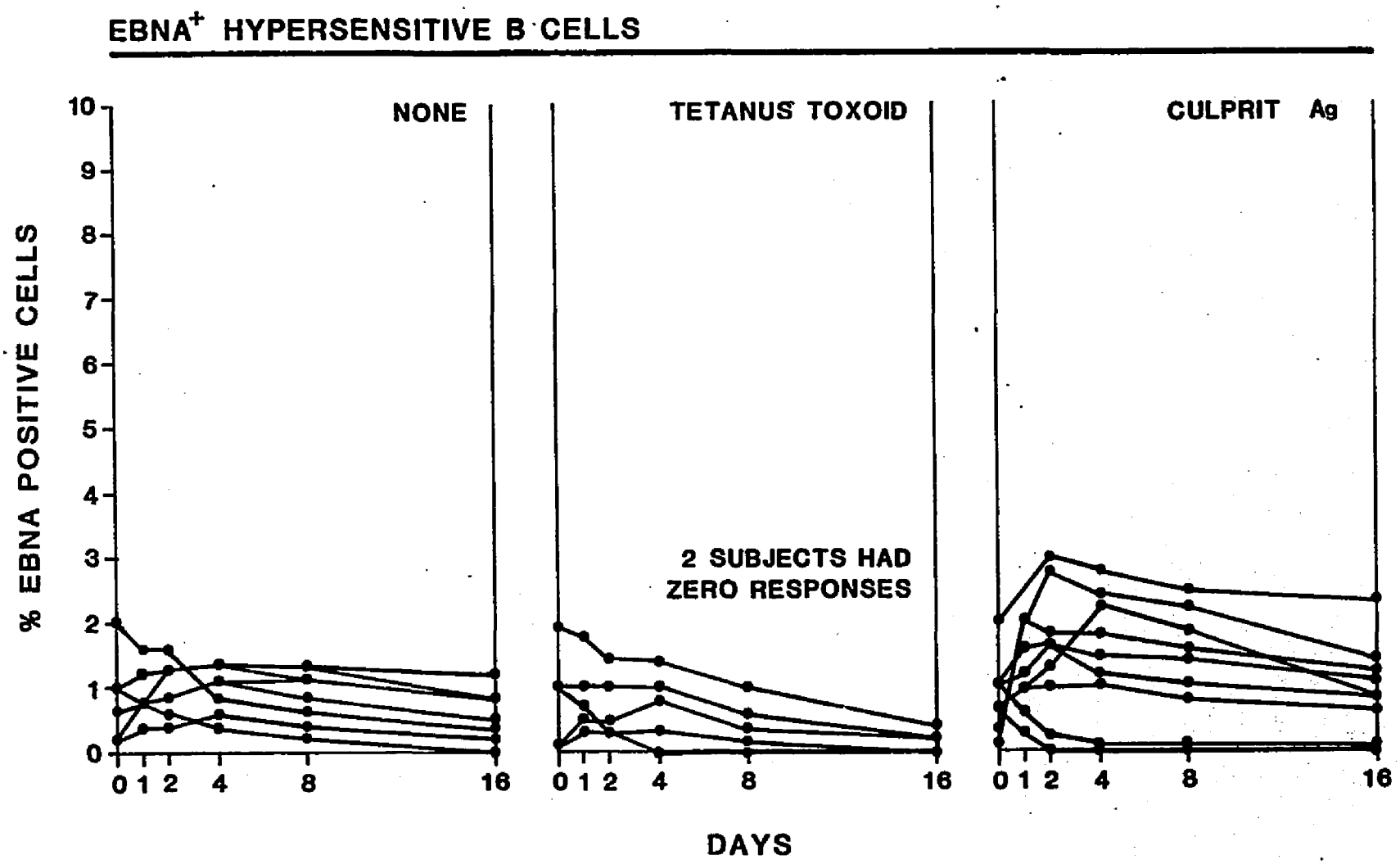

Figure 8. Percent EBNA-positive cells as a function of time in the B-cell cultures from the EBNA-positive hypersensitive subjects when incubated alone, with the tetanus toxoid and with the culprit antigen.

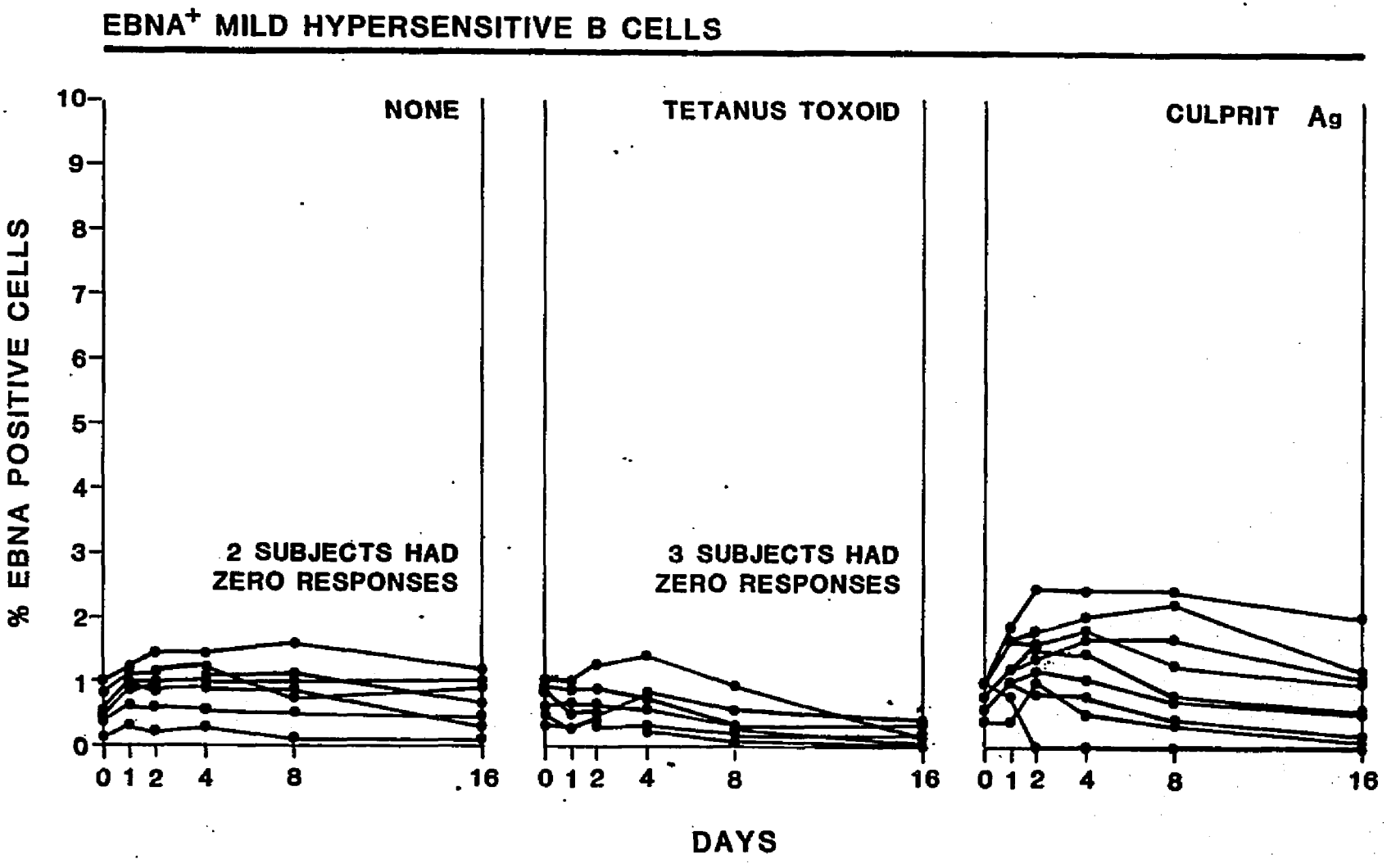

Figure 9. Percent EBNA-positive cells as a function of time in the B-cell cultures from the EBNA-positive mild hypersensitive subjects when incubated alone, with the tetanus toxoid and with the culprit antigen.

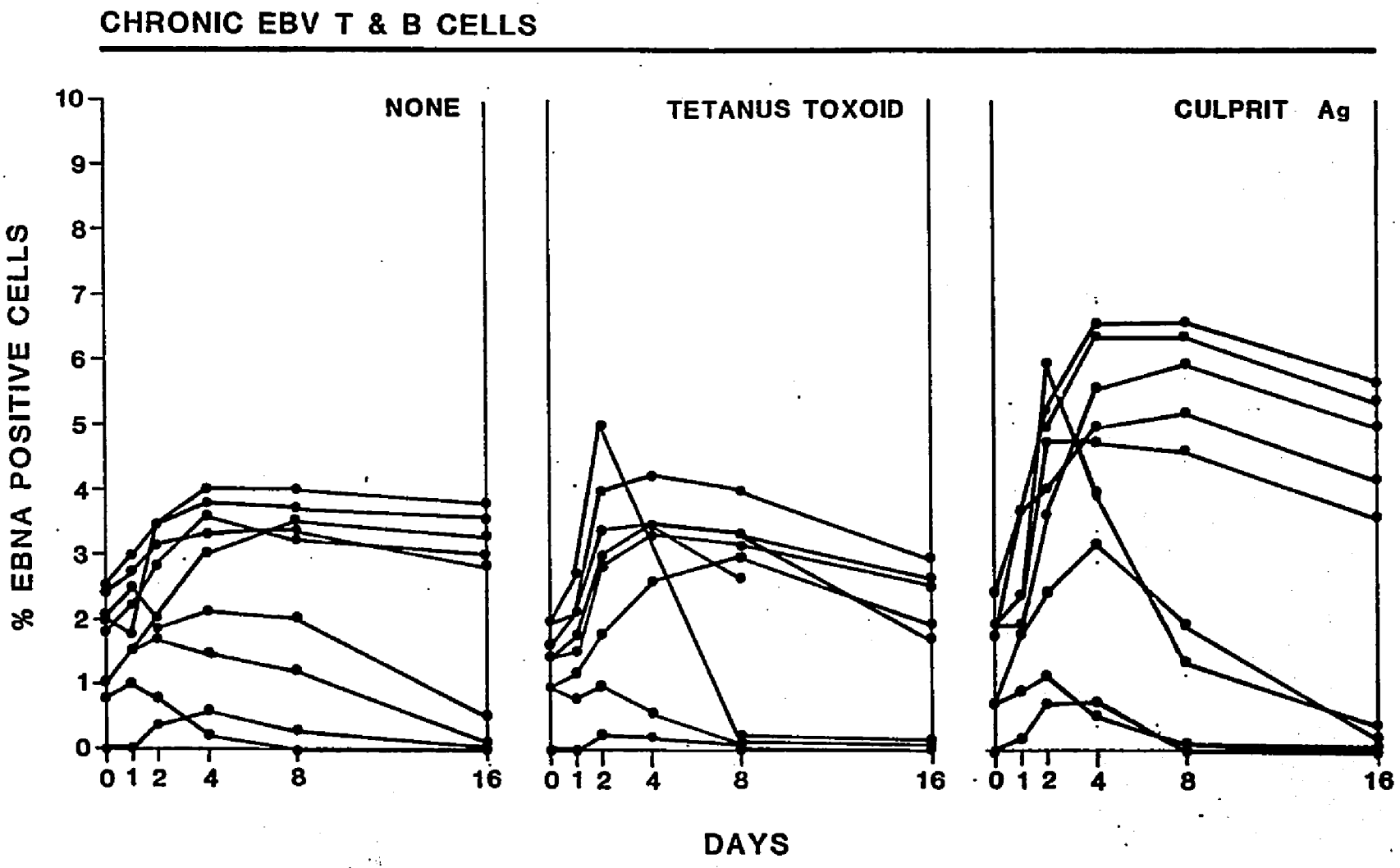

Figure 10. Percent EBNA-positive cells as a function of time in the B- and T-cell cultures from the chronic EBV subjects when incubated alone, with the tetanus toxoid and with the culprit antigen.

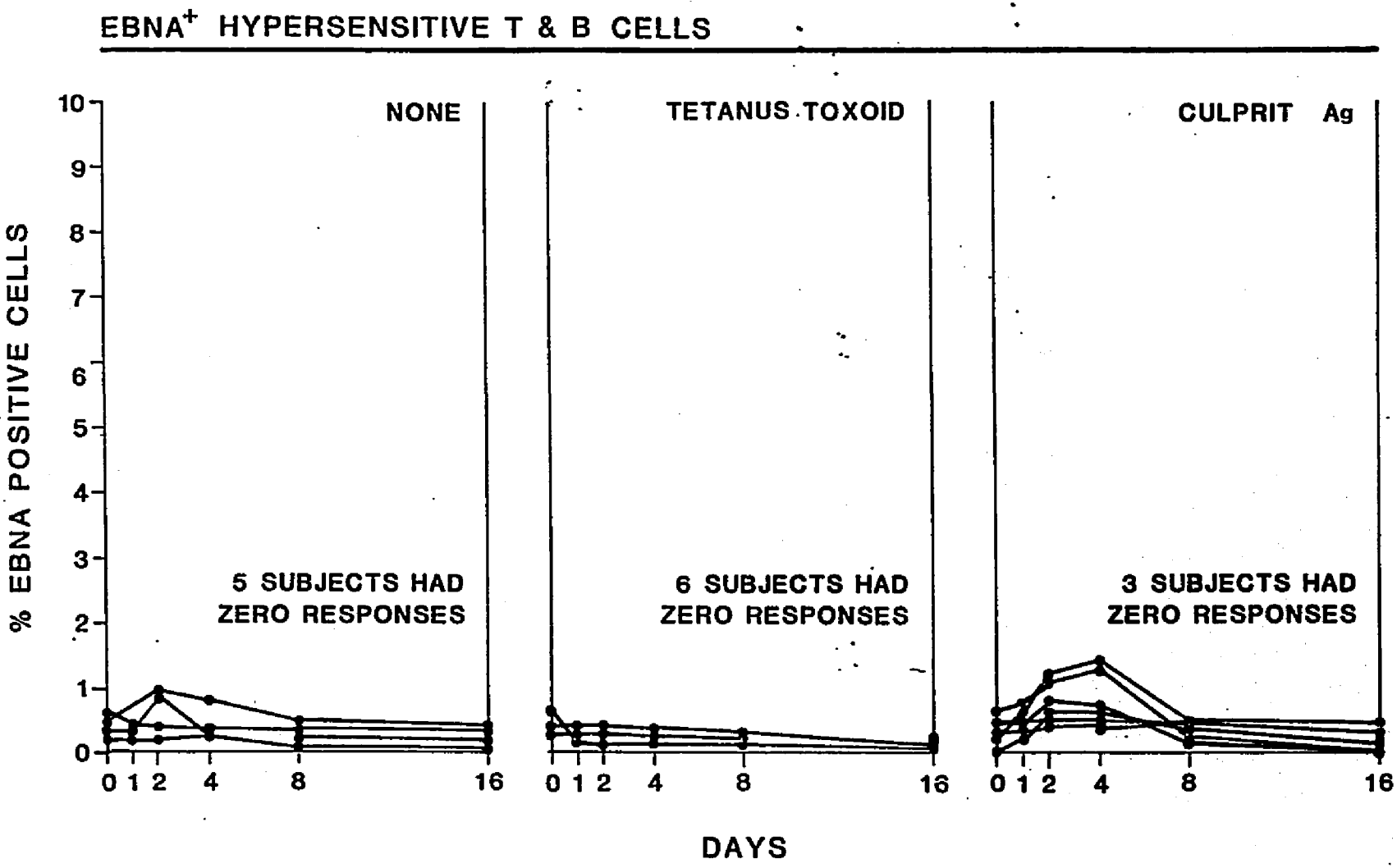

Figure 11. Percent EBNA-positive cells as a function of time in the B- and T-cell cultures from the EBNA-positive hypersensitive subjects when incubated alone, with the tetanus toxoid and with the culprit antigen.

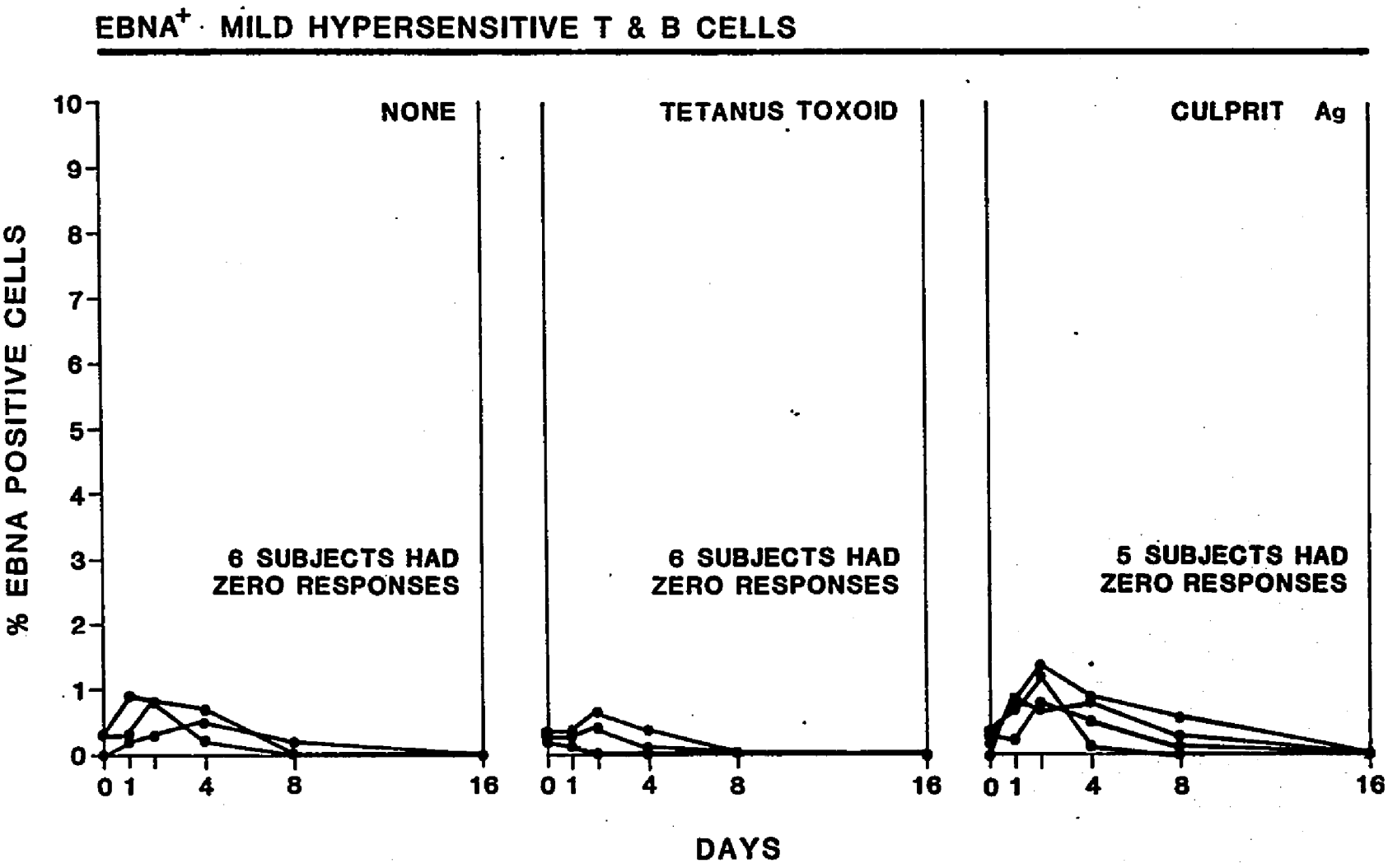

Figure 12. Percent EBNA-positive cells as a function of time in the B- and T-cell cultures from the EBNA-positive mild hypersensitive subjects when incubated alone, with the tetanus toxoid and with the culprit antigens.

In the three groups, cell cultures with the culprit antigens were shown to express an apparent increase of EBNA-positive cells as compared to cell cultures with tetanus toxoid antigen or cell cultures alone. Besides, the number of subjects that failed to generate any EBN-positive cells appeared to increase: (1) as the state of hypersensitivity decreased, (2) in cultures containing B- and T-cells, and (3) in cultures not stimulated with the culprit antigen.

CHAPTER 4

DISCUSSION

The major objective in this research was to investigate a correlation between the presence of hypersensitivity disorders and the inability of the chronic EBV subjects to manage properly the EBV. To achieve this goal, 51 people were chosen in the first analysis to assure placement of at least ten subjects in each of the three experimental groups. Placement into one of three groups was based upon data from the lymphocyte stimulation tests, viral serology and personal clinical histories. The groups include: EBNA-positive hypersensitive ill (chronic EBV), EBNA-positive hypersensitive healthy and EBNA-positive mild hypersensitive healthy subjects.

Many studies have described the incidence and levels of antibody against EBV antigen (9, 32, 53) and the action of suppressor and cytotoxic T-cells against virus altered cells in infectious mononucleosis, and x-linked lymphoproliferative syndrome (10, 13, 33, 34, 38). Comparison of these publications to the results of this study suggests chronic EBV to be different in several ways from infectious mononucleosis, BL and the x-linked lymphoproliferative syndrome. Major findings of this research

revealed that: subjects with chronic EBV: (1) have a higher rate of DNA synthesis in the nonstimulated PBL, (2) can be separated from other subjects with hypersensitivies on the basis of increased anti-VCA and anti-EA titers, (3) have stimulation indices against a battery of allergens that are from two to five-fold greater than other EBNA-positive hypersensitive subjects, (4) have a greater percentage of EBNA-positive cells in PBL population than other EBNA-positive hypersensitive subjects, and (5) develop a greater percentage of EBNA-positive cells following invitro stimulation with the culprit antigens than do cells obtained from other EBNA-positive hypersensitive people.

The contrasting data may be made evident by an abstract of some pertinent events known to occur in other EBV related diseases. Studies of infectious mononucleosis have shown that following primary infection with EBV, the anti-VCA of IgM class and anti-EA appear initially and transiently (9); the virus becomes latent and may persist for the life of the individual in a few B-cells which spontaneously enter into a productive infection (53). These cells synthesize enough MA and VCA but rarely sufficient amounts of EA to maintain production of the corresponding antibodies; however, when an individual becomes compromised or immunosuppressed, high titers of

anti-EA and anti-VCA of an IgG class appear indicating reactivation of EBV. Activation of EBV in a viral carrier results in enhanced production of anti-VCA of IgG class and EA, increased excretion of virus (54, 55) but rarely any signs of illness related to the virus.

In infectious mononucleosis the initial infection of B-cells by EBV is followed by an extensive proliferation of (1) cytotoxic T-cells which are cytotoxic for EBV infected B-cells and (2) suppressor T-cells with specificity towards autologous antigen specific T-cells as well as B-cells immunoglobulin production when stimulated by pokeweed mitogen (34). Furthermore, there is evidence of a transient anergy expressed as a depression in responses to skin test antigens and a hyporesponsiveness of lymphocytes to invitro stimulation of phytohemogglutinin, allogenic lymphocytes and specific antigens (56).

Abnormal function and even absence of function in the subpopulations of T-cells have been reported in the x-linked lymphoproliferative syndrome (57). In one study, Berkel et al. (58) showed that the absence or dysfunction of cytotoxic T-cells (against LYDMA) resulted in a decline of anti-EBNA, whereas the absence or dysfunction of specific suppressor T-cells resulted in elevated levels of anti-VCA and anti-EA titers. Also, mitogenic stimulation

of Raji cells (EBNA-positive non-virus secreting Burkitt's lymphoblastoid cells) caused a marked increase in the expression of EBV early antigen, a decrease in membrane fluidity with no apparant change in cellular DNA synthesis (59).

Data from this study support the hypothesis that chronic EBV represents still another abnormality to be associated with persistent EBV. Chronic EBV displays a different antibody profile, an acute association with hypersensitivity disorder and an apparent difference in the interaction between T- and B-cell population not seen in the three previously described diseases.

Data shown here suggest the chronic EBV subjects have a compromised ability to cope with EBV infections and this defect is manifested in such lymphocyte abnormalities as: (1) PBL which have increased background DNA synthesis, (2) apparent T-cells which show normal or perhaps greater reactivity towards allergens, (3) greater incidence of EBNA positive B-cells with iodiotypic expression for certain allergens, and (4) a greater proliferation of the EBNA-positive B-cells when cultured with designated allergen.

APPENDIX A

Phosphate Buffer Saline (IX):	PBS was prepared by dissolving Nacl 8gm, Kcl 0.3gm, Na_2HPO_4 0.975 gm, KH_2PO_4 0.02gm, and glucose in 1000 ml of D.D H_2O. pH was then adjusted to 7.2. The solution was sterilized by filtration through a 0.45 um Millipore filter and stored at 4°C.
Ficoll-Hypaque Gradients:	Eighteen grams of Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey) were imbibed into 230 ml of double distilled water, 60 ml of Hypaque (Winthrop Laboratories, New York) was then added. The preparation was autoclaved and stored at 4°C until used.
Minimum Essential Media (Eagle):	9.6 gm of MEM (GIBCO, Grand Island, New York) was dissolved in 1000 ml of D.D H_2O, pH adjusted to 7.2 and then the fluid was sterilized by filtration through 0.45 um Millipore filter.

PPO-POP Scintillation Cocktail: Was prepared by mixing 100 ml of toluene with 8gm of PPO (2,5 diphenyloxazole) and 0.45 gm of POPIP (1,4-bis [2-(5-phenyloxolyl)] benzene). The mixture was stirred for approximately two hours on a magnetic stirrer.

Alsever's Solution: Was prepared by dissolving 20.5 gm dextrose, 4.2 gm Nacl, and 8.0 gm sodium citrate in 1000 ml of water. Alsever's solution was then sterilized by membrane filtration through a 0.45 um Millipore filter and stored at 4°C.

Guinea Pig Serum: Was prepared by allowing 30 ml of guinea pig blood to clot, centrifuged at 2500 rpm for 10 minutes and the serum was removed to a sterile vile and stored at -20°C.

UNIVERSITY OF ARIZONA HEALTH SERVICES CENTER

CLINICAL LABORATORIES - VIROLOGY

EB-VCA SEROLOGY

Principle

The indirect fluorescent antibody method is used in the EB-VCA Test. Patient serums are reacted with Burkitt's lymphocytic cells fixed on microscope slides (Gulf Laboratories). If serum antibodies to Epstein-Barr virus are present they will bind to the antigen substrate and not be rinsed off. Subsequently, when antihuman globulin tagged with fluorescein is added to the reaction site, it will bind with the patients' immune antibodies causing the antigenic structures to fluoresce when viewed through a fluorescence microscope.

Reagents

Precaution: For <u>In Vitro</u> Diagnostic Use Only

Preparation and Storage:

1. Phosphate Buffered Saline (PBS): Rehydrate in 1 liter of distilled water. The PBS, which is buffered with 0.1 M phosphate, has a pH of 7.5±.1 and contains 0.01% merthiolate as preservative. The PBS should be stored at 2-8°C.

2. Antihuman Globulin (Caprine), FITC labeled, lyophilized: Reconstitute with 3 ml of PBS. The conjugate is pretitered, contains Evans blue counterstain, and is ready for use. The reconstituted conjugate can be stored 4 weeks at 5°C or aliquoted and stored up to 8 months at -20°C.
3. Positive and Negative Control Sera, lyophilized: Reconstitute with 1 ml of PBS. This gives a 1:10 dilution of the sera. The reconstituted sera are stable for 6 weeks at 5°C or 8 months at -20°C.
4. Mounting Fluid: The mountant is glycerol buffered to pH 8.0. It is ready for use and can be stored at room temperature.
5. Antigen Substrate Slides: The viral antigen slides are immediately ready for staining. The antigen slides consist of HRIK Burkitt's lymphocytic cells in which the percentage of cells demonstrating virus has been enhanced to about 5% to permit easy reading and optimal contrast. The slides have been fixed and are noninfective. The slides stored at 2-8°C are stable until the date indicated on the slide package label.

Specimen Collection and Preparation

Blood obtained by venipuncture should be allowed to clot and then centrifuged. The serum should be separated and refrigerated or stored frozen if not tested in the same week.

Instructions For Use of Kit

The entire kit should be stored in the refrigerator. The kit is ready for use after reconstitution of reagents. Individual slide packets should only be opened just before use.

Material Provided

1. Epstein-Barr Virus Antigen Slides, 10 slides with 10 wells each.
2. Antihuman Globulin, FITC labeled, 3 ml working solution containing Evans blue counterstain.
3. EBV Positive and Negative Control Sera, 1 ml each
4. PBS Powder, 1 liter
5. Mounting Fluid, 3 ml

Test Procedure

1. Fill out worksheets.
2. Dilute positive control through 1:1280 in PBS. NOTE: Reconstituted control is 1:10.
3. Dilute patient serum 1:10 through 1:2560 in PBS.

4. Remove one slide for each patient serum plus one control slide.
5. Using a Pasteur pipet, add just enough approximately diluted serum to cover each reaction site. Use 1 slide for positive control titration and 1 well for negative control.
6. Place slide in a moist chamber and incubate at room temperature for 30 minutes.
7. Rinse slide briefly with a gentle stream of PBS and then rinse 5 minutes in PBS. Don't rotate.
8. Remove excess PBS.
9. Add just enough conjugate to cover each reaction site.
10. Incubate slide at room temperature in a moist chamber for 30 minutes.
11. Rinse and remove excess PBS as in 7 and 8.
12. Using glycerol mountant, coverslip.

    NOTE: Place positive control at 1:10 and negative control at 1:10 on each slide.
13. Read slide as soon as possible at 150X to 200X magnification using fluorescence microscope.

Quality Control

Each kit contains positive and negative control sera which should be incorporated into each testing run. The negative control must be negative at the screening dilution. The positive control serum is titered to provide a standard for checking test sensitivity. It should exhibit a 4+ reaction at the screening dilution and diminish to a 1+ reaction at its titer dilution. A day to day variance of one 2-fold dilution on either side of the stated titer is acceptable performance.

Results

Since approximately 5% of the cells express the virus, the reaction is POSITIVE when 5% of the cells in each field exhibit a greenish-yellow fluorescence. The remaining lymphocytes provide a red contrasting background.

The reaction is NEGATIVE when cells do not fluoresce greenish-yellow but appear red due to the counterstain.

The EBV titer is the highest dilution of serum which produces a 1+ fluorescence in some of the cells. A titer less than 1:10 indicates susceptibility and a titer of 1:10 or greater indicates immunity to infectious mononucleosis.

Test Limitations

Antinuclear antibodies present in serum may interfere with the EBV Test. However, the diffuse, reticular or peripheral staining produced by the antinuclear antibodies is easily distinguished since it is dull and involves most of the cells. The homogeneous staining of EBV antibody involves only 5% of the cells.

Expected Values

In classical infectious mononucleosis antibodies to Epstein-Barr virus develop early, reach peak titers within a few weeks, and then decline to lower levels which persist indefinitely. Measuring a 4-fold rise in titer is diagnostic of active disease. However, since peak titers are often reached before the taking of an acute phase serum, a single serum titer of 320 is strongly suggestive and a titer of 640 is definitive of active or recent EBV infection. Females and tonsillectomized children develop higher titers to Epstein-Barr virus than their respective male and nontonsillectomized counterparts. Higher titer levels are also developed by persons with histories of pneumonia or urinary tract infections. A later rise in titer, which can exceed 2560, is the result of secondary disease, such as Burkitt's lymphoma or nasopharyngeal carcinoma. The titer of antibodies to Epstein-Barr virus, as with heterophile antibodies, does not reflect the

severity of clinical symptoms in infectious mononucleosis. See EB titer correlations.

Performance Characteristics

Results of the indirect fluorescent antibody test for Epstein-Barr virus parallel those of complement fixation test, but do not correlate with the heterophile antibody test. In EBV infectious mononucleosis heterophile antibody fails to develop in about 10% of adults, more frequently in children, and almost uniformly in infants. The development of EBV antibody occurs in all EBV infections and is not affected by age. Heterophile antibody is short lived; whereas EBV antibody persists indefinitely.

Antibodies to VCA as measured in the indirect fluorescent antibody test are a dependable indicator of immunity and can serve to diagnose active disease. The antibody titers are not affected by other febrile viruses, such as coxsackievirus, adenovirus, myxovirus, and other herpes viruses. Results are reproducible within one tube dilution.

EB VIRUS EARLY ANTIGEN SEROLOGY

Principle

This test is for detection of antibody to Epstein-Barr (EB) virus early antigen by indirect immunofluorescence. Antibodies to early antigen (EA) are present as a result of active replication of EB Virus. This may either be due to acute or reactivation infection.

Materials

1. EB-EA slides containing 10 wells of EB-Ea positive NC37 cells.
2. FITC-conjugated Goat antihuman IgG (H+L) (working dilution of 1:25 - 1:100) (Store at -60°C in .1 ml aliquots)
3. Evans Blue in PBS.
4. PBS (Phosphate Buffered Saline)

   0.15 M NaCl in 0.10 M phosphate buffer, pH 7.5

Na_2HPO_4 (anhydrous)	1.236 gm
$NaH_2PO_4.H_2O$	0.18 gm
NaCl	8.5 gm
Distilled water q.s.	1000.0 ml

5. Mounting Fluid, Buffered glycerin pH 7.5

PBS pH 7.5	1 volume
Glycerin (reagent grade)	9 volumes

6. Positive and Negative Control sera (aliquot in 100µl samples and store at -60°C).

Specimen Collection

Test sera should be promptly removed from clotted blood and stored at -20°C or below until day of testing.

Procedure

1. Remove one 10-well EB-EA test slide from the freezer for each 2 samples plus one for a control slide. Allow to warm to room temperature before opening.
2. Make out worksheet and identify slides with appropriate labeling. Be sure to record the slide, conjugate and control lot numbers.
3. Make fresh dilutions of the test and positive control sera as follows:
   a. 1:20 (0.1 ml serum + 1.9 ml PBS).
   b. Serial dilutions of 1:20 dilution to 1:320 (two-fold dilutions).
   c. Negative control serum needs only to be used at 1:20. Use also a PBS control on one well of control slide.
4. Place each slide with well side up in humidified chamber.

5. Using a micropipette or pasteur pipette, place 15-25 µl of diluted serum or PBS on appropriate well. The same pipette may be used for all dilutions of a serum if the order of pipetting is from the highest to the lowest dilution.
6. Incubate at 35°C in humidified chamber for 30-60 minutes. Be careful in handling the chamber to avoid allowing the sera from running together.
7. Remove slides from humidified chamber and gently rinse each slide with PBS from a wash bottle. Be careful not to direct stream of PBS directly on wells.
8. Wash for 2 five-minute PBS washes in staining dish.
9. Drain excess PBS from slide.
10. Return to humidified chamber.
11. Cover wells with FITC conjugated goat anti-human IgG at appropriate dilution.
12. Incubate in humidified chamber at 35°C for 30-60 minutes.
13. Repeat steps 7-9.
14. Counterstain with Evans Blue for 1 minute.

15. Mount slide with buffered glycerol and coverslip. Drain off excess mountant onto terri towel.
16. Read with FA scope using 40X objective. If slides cannot be examined immediately, store at 2-8°C in a light-tight container.

Reading of Slides

Sera containing antibody to EB-EA will demonstrate lime-green fluorescence on the EB-EA positive cells. Only 30-80% of the cells should be EB-EA+. If all cells fluoresce, check for ANA. If problems consult your supervisor.

Interpretation

While a positive EB-EA serology is indicative of active viral replication, the results should be considered with the VCA and EBNA serologies before a final interpretation can be made. The patient's clinical history must also be considered.

In General

VCA	EBNA	EBEA	Consistent with
Positive	< 2	positive	acute infection
Positive	≥ 2	negative	past infection
Positive	≥ 2	positive	active infection
Positive	negative	negative	acute infection

EBNA SEROLOGY

A. Slides

Use 12-well Bellco slides

B. Preparation of Cells

1. Raji cells are used for EBNA.
2. Cells which are ready to be split are harvested by centrifuging at 500-1000 rpm for five minutes to pellet cells. Media should be discarded in dirty discard pan.
3. Cells should be resuspended in PBS without phenol red and washed once in PBS $\overline{s}$ phenol red. Washing is accomplished by resuspending in fresh PBS $\overline{s}$ phenol red and repelleting. After washing, resuspend cells in fresh PBS $\overline{s}$ phenol red. Some of the clumps of cells may not readily break up. These should be allowed to settle out, using only the single cell suspension.
4. Dilute cells to make a <u>slightly</u> cloudy suspension and drop onto wells on the slide made above. Drops should not be so large as to become confluent. Allow to dry. Use of 25 µl dropper may be useful.
5. Fix in -20°C acetone for 10 minutes.
6. Allow to dry.

7. Store at -60°C.

C. Preparation of Serum

1. Blood should be aseptically collected, allowed to clot, centrifuge at 2,500 for 10 minutes, and serum removed to a sterile 1 dram vial.
2. If not to be run within 24 hours, sera should be stored at -20°C or lower. If paired sera are to be collected, both specimens should be run at once.
3. Mix serum gently to avoid layering due to refrigeration or freezing.
4. Dilute in PBS without phenol red pH 8.0 to the following dilutions.

   1:2 to 1:32

5. Dilute positive control as patient serum.
6. Dilute negative control 1:2.

D. EBNA

1. Overlay positive and negative controls and diluted patient's serum in appropriate wells.
2. Incubate in humidified chamber at 35°C for 60 minutes.
3. Wash in 2-five minute washes in PBS.
4. Overlay with guinea pig complement. (This should be stored at -70°C until used) and aliquoted out so that only the portion to be used is thawed. This is fresh guinea pig serum which is overlaid

as a source of complement. Dilution is noted on vial or box.

5. Incubate in moist chamber at 35°C for 60 minutes.
6. Wash in PBS for two (5) five minute washes.
7. Overlay with anti-guinea pig complement conjugate and incubate in moist chamber at 35°C for 60 minutes.
8. Wash as in #3 and 6. Mount and coverslip. $\geq$ 80% should express EBNA and be fluorescent positive in this test. The pattern is one of speckled nuclear fluorescence.

E. Sources of Problems

1. Use of unclean slides will give nonspecific fluorescence.
2. Failure to completely overlay sera, complement or conjugate over the slides will cause false negatives.
3. Incomplete washing at any stage can cause false positives. This is controlled with negative control.
4. Overwashing can cause false negatives.
5. Too thick a cell layer can cause a loss of reactive cells during washing.
6. Inactive conjugate can cause false negatives but this is controlled by the positive and negative controls.

7. Drying of slide at any step can cause false positives. This can be prevented by overlaying complement and conjugate with parafilm during incubation.

F. Interpretation

1. VCA - Positive

   EBNA - <2

   consistant with acute infection (ACUT)

2. VCA - Positive $\leq$ 160

   EBNA - $\geq$ 2 Positive

   consistent with past infection (CPI)

3. VCA $\geq$ 320

   EBNA - $\geq$ 2

   Do EBEA or IgM VCA. If positive - consistent with active infection (CAI). If negative, consistent with past infection.

4. VCA - Negative

   EBNA - Negative

   Report as NO EB.

5. If four-fold or greater change in titer between acute and five convalescent sera, this is usually consistent with acute or active infection. If VCA rises or remains stable, and EBNA goes from negative to positive, it is an acute infection.

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