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AN IMMUNOPRECIPITATION BLOCKING ASSAY FOR THE ANALYSIS OF EBV INDUCED ANTIGENS

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A technique for the analysis of EBV antigens in extracts of unlabelled EBV infected cells has been developed. Using this technique we have demonstrated that the EBV early antigen complex consists of several proteins and is not completely expressed in chemically induced Raji cells. Studies with a range of sera from patients with infectious mononucleosis and nasopharyngeal carcinoma have shown that despite similar titers by the indirect fluorescent antibody test different populations of proteins were precipitated by different sera.

immunoprecipitation inhibition EBV antigens EBV serology EBV titration

INTRODUCTION

Immunoprecipitation, either direct or indirect, has proven to be a powerful technique for studying virus specific proteins. When monoclonal or highly specific antibodies are available the immune precipitates are of high quality exhibiting little if any non-specific precipitation of host proteins. A major problem when studying many viruses is that the antisera available are usually obtained from patients in the acute phase of the disease, and such sera contain appreciable levels of antibodies to proteins unrelated to the virus (eg. heterophile antibodies, antibodies to DNA, antibodies to many intracellular proteins). Immune precipitates prepared using patients sera will be considerably contaminated with proteins not coded for by the virus genome. A second major problem is that the normally low titers of the antibodies of interest preclude direct immune precipitation and necessitate the use of an indirect technique. This leads to a third problem in that many proteins bind directly to the solid phase used to precipitate the immune complexes; in addition, several proteins bind specifically to immune complexes and measures should be taken to prevent this interaction. For several reasons it has become desirable to prepare certain Epstein-Barr virus (EBV) antigens in large quantities, often from sources which are not amenable to radiolabelling, and a method is required to follow these antigens through a purification scheme. In order to overcome these problems we developed a technique using a standard extract of radiolabelled EBV antigens and a pool of standardized serum which can be

preabsorbed with the extract of interest before use for precipitating the radiolabelled proteins. A modification of this technique allows the analysis of antigens present in extracts containing large amounts of IgG which would normally compete with the antigen/antibody complexes for the solid phase.

MATERIALS AND METHODS

Tissue culture and virus production

P3HR1 cells, which produce infectious EBV, were grown in RPMI 1640 media supplemented with 10% heat inactivated newborn calf serum. When the cultures reached a density of 10^6 cells/ml they were diluted with an equal volume of fresh medium. For induction of EBV antigens and to increase the virus yield cultures were treated with phorbol ester (TPA, 40 nM) and sodium butyrate (3 mM) immediately after subculture and then incubated at 33°C for 7 to 10 days. The culture medium was clarified by centrifugation at 4,000 × *g* for 15 min and concentrated using a pellicon ultrafiltration cassette with a nominal cut off value of 100,000 daltons. The concentrated medium was centrifuged at 27,000 × *g* for 2 h at 4°C to precipitate the virus, which was subsequently resuspended in a small volume of tissue culture medium and stored at −138°C.

Raji cells which carry EBV in a latent state were cultured similarly. The endogenous genomes were induced with TPA and butyrate as above, but only for 48 h. BJA cells which are EBV genome negative were also cultured in a similar manner.

EBV titration

The definition of the infectivity of stocks of EBV presents a problem. The efficiency of replication of the virus depends on: (1) the multiplicity of infection (MOI); (2) the metabolic state of the target cells; and (3) the cell type used to support the infection. In addition, the induction of stainable EA in superinfected Raji cells is not a good criterion; when very high MOI's are used the cells are so loaded with virus capsid antigen (VCA) introduced by the infecting virions that it is not possible to distinguish newly synthesized antigen from passively adsorbed antigen. For this reason each virus stock is tested for its ability to induce the synthesis of certain immune precipitable EBV proteins in Raji cells: 1 Raji cell superinfecting unit (RCSU) is capable of inducing the synthesis of EBV tertiary proteins in 10^6 Raji cells.

Preparation of extracts for immunoprecipitation

Cells, either radioactively labelled or not, were washed twice with Hanks' phosphate buffered saline (PBS) to remove serum contaminants. The cells were resuspended in IP buffer (137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 10% glycerol, 0.01% NaN_3, 1 μg/ml PMSF, 20 mM, Tris-HCl pH 9), disrupted with ultrasound and incubated on ice for 30 min. The extracts were then centrifuged at 100,000 × *g* for 30 min at 4°C in a TFT 65 rotor (Kontron). For volumes of 175 μl or

less, we used the Beckman Airfuge, at maximum speed for 5 min. The clarified supernatants were either used immediately or stored at -20°C until required. For unlabelled extracts we used 10^8 cells/ml IP buffer, for radioactively labelled cells 10^7 cells/ml IP buffer. Extracts of cells prepared using other buffers, or fractions from various purification procedures, were either adjusted to the correct concentrations of the various components of IP buffer or were dialysed against IP buffer (100 vols., 2 changes overnight).

Preparation of the solid phase for indirect immunoprecipitation

Originally we used formalinized *Staphylococcus aureus* Cowan 1 strain prepared according to Kessler (1975). However, subsequent experiments indicated that purified protein A attached to Sepharose beads (Pharmacia) was easier to handle, gave cleaner precipitates and the polyacrylamide gels prepared from such precipitates were of a higher quality. The protein A beads were weighed (3 mg beads/10 µl serum used) and swollen for 1 h in IP buffer. After washing the beads were resuspended at a concentration of 3 mg beads/100 µl IP buffer.

Adsorption of human sera

Ten µl of a standard pool of EBV positive sera (EBV nuclear antigen, EBNA 1 : 400, EBV early antigen, EA 1 : 1200, VCA 1 : 6600, the titers were determined using standard immunofluorescent techniques, EBNA (Reedman and Klein, 1973), EA (Henle et al., 1970), VCA (Henle and Henle, 1966)) were mixed with 100 µl of an extract prepared from EBV-negative BJA cells (10^8 cells/ml buffer) and if required an aliquot equivalent to 10^7 antigen-positive cells. The mixture was then incubated overnight at 4°C. For sera with different titers, the amount of cold, antigen containing extract, required to block the serum must be determined by titration.

Preparation of the immunoprecipitates

The adsorbed serum was mixed with 100 µl of the protein A bead suspension and gently shaken at room temperature for 30 min. The beads were then collected by centrifugation, and 100 µl of an extract of superinfected Raji cells labelled with [^{35}S] methionine (Bayliss and Wolf, 1981) were added to the antibody coated beads. The mixture was further shaken for 2 h. The beads were collected by centrifugation, washed 3 times in IP buffer and twice in distilled water. The immune complexes were eluted by heating the beads in polyacrylamide gel electrophoresis sample buffer (50 mM Tris-HCl, pH 7.0; 2% SDS; 5% 2-mercaptoethanol; 30% sucrose) to 100°C for 5 min. The mixture could be directly analysed on polyacrylamide gels without the need to remove the sepharose beads.

SDS polyacrylamide slab gel electrophoresis (PAGE)

The resolving gel contained 10% acrylamide, 0.26% diallyltartadiamide (DATD), 0.1% SDS and 0.375 M Tris-HCl pH 8.5, the gel length was 15 cm; the stacking gel

contained 3% acrylamide, 0.08% DATD, 0.1% SDS and 0.12 M Tris-HCl pH 7.00. Polymerization was initiated by adding ammonium persulphate and *N, N, N'*, N'-tetramethylenediamine (TEMED). The upper and lower buffer tanks contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS (pH 8.5). Electrophoresis was carried out at room temperature using a constant current of 5.3 mA/cm^2 and continued until the leading front reached the end of the gel. The gels were fixed and stained for 1 h in a mixture of water, acetic acid and isopropanol (4 :1 : 1) containing 0.1% Coomassie brilliant blue, destained in a mixture of water, acetic acid and isopropanol (8 : 1 : 1) and dried onto Whatman 3 M filter paper under vacuum. Either Kodirex X-ray film or LKB 2208 Ultrofilm ^{3}H was used to prepare autoradiograms of the dried gels. The apparent molecular weights of the proteins were estimated by comparison with a set of standard proteins. Approximately 200 ng of each polypeptide were applied per slot.

RESULTS

Standardization of the serum pool used for immunoprecipitation

Using the immunoprecipitation method described above we tested over 100 human sera for their reactivities against EBV specified proteins extracted from superinfected Raji cells (Raji SI). Some correlation between the fluorescent antibody staining technique (FA) titers against the FA-defined antigens and the proteins precipitated by the sera were obtained. However, there were a number of inconsistencies, particularly

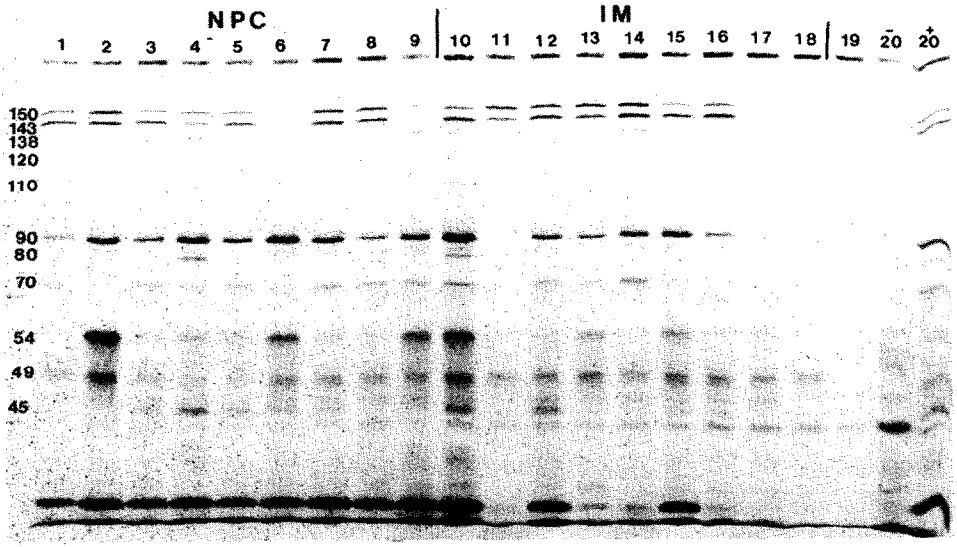

Fig. 1. Immunoprecipitates prepared using various sera from NPC and infectious mononucleosis patients. Using the immunoprecipitation method described in the text the sera were used to prepare precipitates from extracts of superinfected Raji cells which had been labelled from 12 to 16 h post-infection. The titers of the sera as determined by the indirect FA test are given in Table 1. Serum 19 is a serum obtained from an EBV negative individual. Serum 20 (a standard pool of NPC sera) was used to prepare precipitates from uninfected (-20) and infected (+20) Raji cells. The apparent molecular weights are given as daltons $\times 10^{-3}$.

clear examples of this are proteins 138 and 80 of Fig. 1. This inconsistency has led to discrepancies in work published by various laboratories. Figure 1 illustrates this point with sera taken from patients with nasopharyngeal carcinoma (NPC) and infectious mononucleosis (IM). The most dramatic inconsistencies can be seen with sera 17 and 18, which precipitate almost none of the proteins above a molecular weight of 54 kD whereas sera 1 and 2 (which have FA titers similar to 17 and 18) precipitate a large number of proteins. (See Table 1 for the titers against the various antigens.) In order to overcome the problem of variability, we mixed several high titered sera from NPC patients to give a serum pool having the following titers: anti VCA 1 : 6,600, anti EA 1 : 1,200, anti MA 1 : 320 and anti EBNA 1 : 400. Figure 2A shows the spectrum of proteins precipitated by this pool of serum from uninfected Raji cells and infected Raji cells labelled early or late after infection.

TABLE 1

Titers of the sera used to prepare the immunoprecipitates shown in Fig. 1

Track no.	Serum no.	Titer	
		Anti-EA	Anti-VCA
1	Ull	256	1024
2	11118	256	1024
3	11560	256	256
4	11977	2048	2048
5	12197	1024	4096
6	397	1024	16384
7	408	256	2048
8	1151	256	256
9	1811	2048	8192
10	1897	1028	5128
11	1909	320	1280
12	3232	512	8192
13	2094	640	5120
14	2144	320	1280
15	2065	320	640
16	4663	256	1024
17	9327	512	1024
18	9991	256	1024
19	ND	0	0
20	P1	1200	6600

The titers of the sera are expressed as the reciprocal of the dilution of the serum which just gives a positive signal in the indirect fluorescent antibody test. As antigen for EA-acetone fixed IUdR-induced Raji cells were used, for VCA acetone/methanol-fixed phorbol ester/sodium butyrate-induced P3HR1 cells. Serum Ull was a gift from G. Lenoir, sera 1897, 1909, 2094, 2144, 2065 were provided by W. and G. Henle. The remaining sera were obtained from the diagnostic laboratory of our Institute.

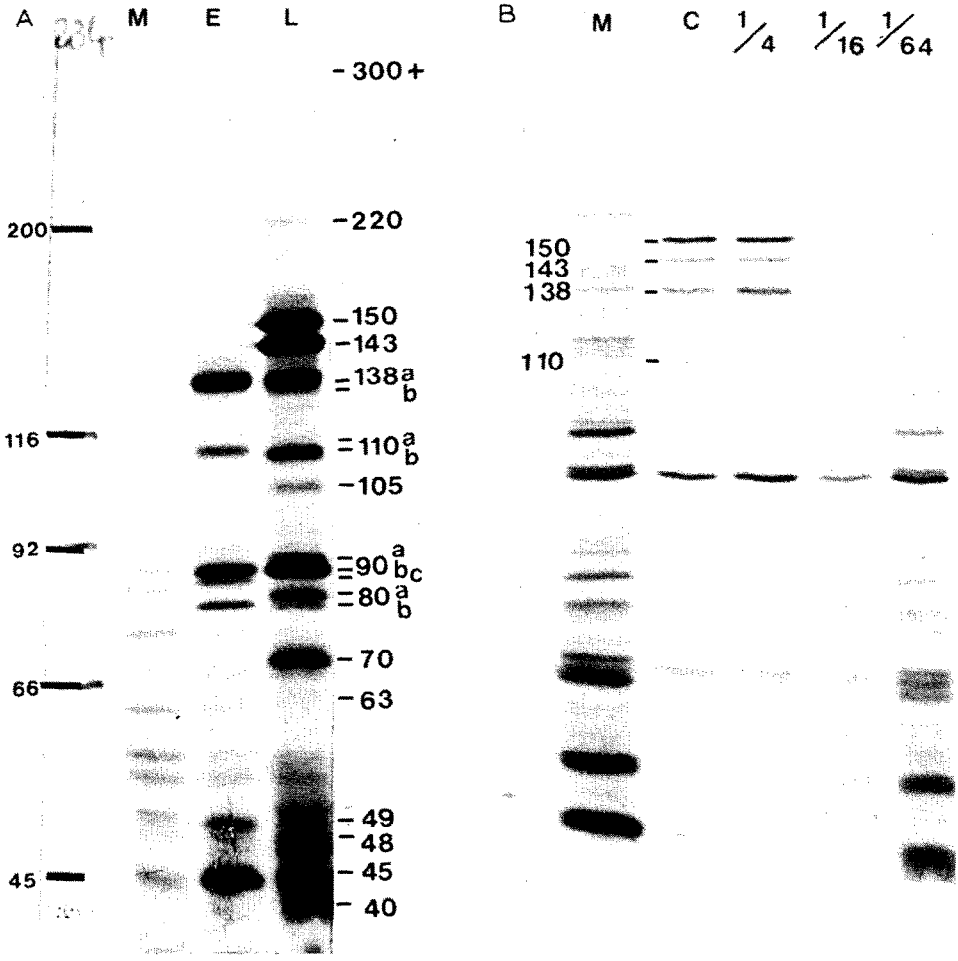

Fig. 2. (A) Immunoprecipitates prepared from M: uninfected Raji cells, E: SI Raji cells labelled from 3 to 7 h post-infection, L: SI Raji cells labelled from 12 to 16 h post-infection. The molecular weights of the EBV proteins are given to the right of the profiles, those of the standard proteins to the left. (B) Titration of a stock of EBV. M: uninfected Raji cells. C: Raji cells superinfected with undiluted virus. 1/4, 1/16, 1/64: Raji cells infected with dilutions of the virus in tissue culture medium. 0.25 ml of virus was used to superinfect 0.5 $\times 10^6$ Raji cells. The cultures were incubated at 37°C for 12 h and then labelled with [^{35}S] methionine for 4 h. After disruption in PAGE sample buffer the extracts were analysed by SDS-PAGE as described in the text. 150, 143, 138, 110 are major EBV specified proteins.

Standardization of the extract of superinfected Raji cells used for immunoprecipitation studies

When Raji cells are superinfected at high multiplicities with EBV derived from P3HR1 cells they synthesize a large number of EBV specified proteins (Bayliss and Nonoyama, 1978, Wolf and Bayliss, 1978, Bayliss and Wolf, 1981). The success or failure to achieve a good superinfection is highly dependent upon the quality of both

the virus concentrates and the target cells. Generally speaking, we have found that a simple titration of EBV by superinfection of Raji cells followed by FA staining with antisera having titers against EBV determined antigens is not a satisfactory method. Preparations of virus having similar titers by the FA test often give quite disparate results when used for preparation of labelled extracts for polyacrylamide gel analysis (PAGE). Thus, we found it necessary to titrate virus stocks by superinfection followed by labelling with [^{35}S] methionine between 12 and 16 h after infection. After extraction, as described above, the proteins were analysed by SDS-PAGE. An example of such an analysis is illustrated in Fig. 2B. Note that some proteins are synthesized even at the 1 : 64 dilution (eg. 138), whereas others are only synthesized in cultures infected at higher MOI's (eg. 110). A second important factor is the inhibition of host cell synthesis, at the 1 : 64 dilution of the virus stock host protein synthesis was hardly affected whereas at lower dilutions almost no host proteins can be observed in the profiles. Figure 2A illustrates a typical immunoprecipitation experiment using superinfected Raji cells and the pool of serum described above. Note the relatively low host background (track M-uninfected Raji cells, E Raji cells superinfected with EBV labelled from 3 to 7 h post-infection and L Raji cells superinfected with EBV labelled from 12 to 16 h post-infection). The various EBV specified or induced proteins are labelled with their approximate molecular weights in kilodaltons. From the data shown in Fig. 2A, we decided to use cells labelled from 12 to 16 h post-infection in all subsequent experiments.

The blocking of the precipitation of specific proteins by absorption of serum with extracts of antigen-positive cells

Extracts of unlabelled cells were prepared by sequential extraction with distilled water, PBS, PBS + 1% NP40 and finally IP buffer. BJA cells are EBV-negative B-lymphoblastoid cells, Raji cells express a single antigen EBNA, Raji cells induced by incubation for 3 days in the presence of 3 mM butyric acid and 40 ng/ml phorbol ester contain early antigens (30% of the cells were positive in the FA test) and P3HR1 cells contain all EBV antigens (10% of the cells were positive in the FA test). Ten µl of the pooled serum were incubated with 100 µl of the extracts overnight at 4°C and the absorbed sera were then used to prepare immunoprecipitates from an extract of labelled SI Raji cells.The blocking index for each extract is given in Table 2 and a polyacrylamide gel analysis of the precipitates is presented in Fig 3. Note that this autoradiogram was heavily over-exposed to detect any residual precipitating activity. Although complete blocking is not always achieved, the dramatic quantitative reduction in the density of the bands on the autoradiogram is certainly sufficient to allow conclusions about the presence of the various proteins within the extracts. The data presented show that a simple aqueous extract of induced Raji cells contains all of the extractable proteins. Similarly, the extract of P3HR1 contains most of the antigens (a notable exception is the upper band of the 80 complex). Sequential extraction of the cells with PBS and detergent containing buffers shows that some of the antigens are

TABLE 2

Blocking indexes of the extracts used in the experiment illustrated in Fig. 3

Extraction buffer	Blocking index of extracts of		
	Raji cells	Ind. Raji	P3HR1 cells
H_2O	0	33	78
PBS	8	13	78
PBS/NP40	0	10	75
IPB	13	17	79

$$\text{Blocking index} = 1 - \frac{\text{immune precipitable counts with absorbed serum}}{\text{immune precipitable counts with BJA absorbed serum}} \times 100.$$

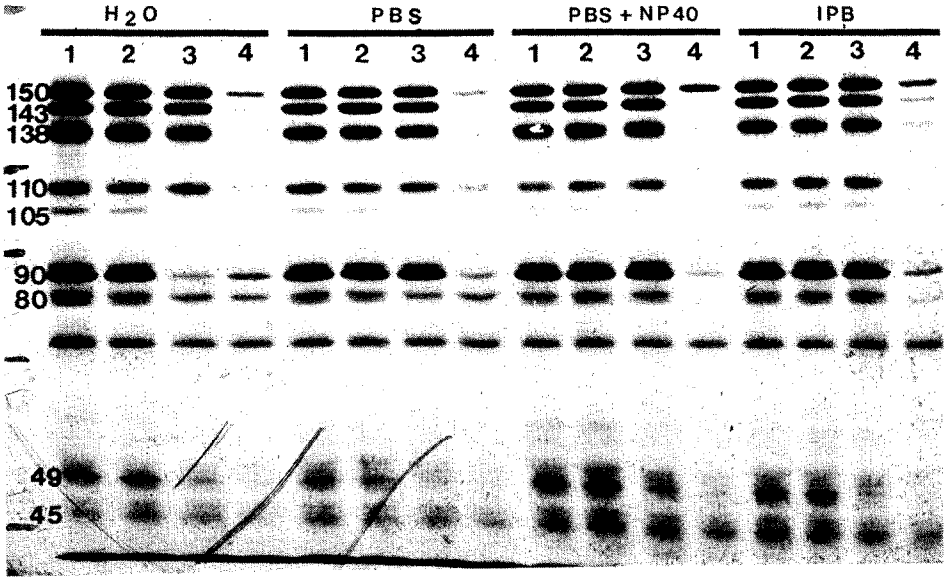

Fig. 3. Immunoprecipitation blocking assay. Extracts of BJA (1), Raji (2), Induced Raji (3) or P3HR1 (4) were prepared by sequential extraction with distilled water (H_2O), phosphate buffered saline (PBS), PBS containing NP40, immunoprecipitation buffer (IPB). The extracts were used to preabsorb a pool of NPC sera which was then used to prepare immunoprecipitates of an extract of ^{35}S-labelled SI Raji cells. The numbers to the left of the gels are the apparent molecular weights in daltons $\times 10^{-3}$.

more efficiently extracted by detergents. Consider protein 150: this protein is efficiently eluted from the cells with PBS since subsequent extraction with the detergent containing buffers fails to release additional amounts of this protein. Protein 143, on the other hand, is a relatively insoluble protein. Even during the last extraction step sufficient quantities of this protein are eluted from the pellet to block the precipitation

of the labelled protein. Such proteins may represent membrane or membrane bound proteins.

Immunoprecipitation blocking by extracts containing high concentrations of serum

It is often of interest to examine proteins which are released into tissue culture medium by infected cells. This presents a particular problem when using the indirect immunoprecipitation method since the IgG from the calf serum used in the tissue culture medium competes with the IgG present in the antiserum for the binding sites on the protein A sepharose. To circumvent this problem the following method was adopted. The human serum is first adsorbed with the negative cell extract as described above and then allowed to react with protein A beads, the IgG coated beads are then mixed with the serum containing extract at room temperature for 2 h or overnight at 4°C. Following the absorption, the standard labelled extract is added and the test continued as before. An analysis of proteins present in a partially purified sample of P3HR1 culture medium is presented in Fig. 4. The clarified medium from 10 l of P3HR1 cells was concentrated to 250 ml using a pellicon membrane cassette system with a nominal molecular weight 'cut off' value of 100,000 daltons. After centrifugation at 27,000 × *g* for 2 h to pellet the virions the supernatant was brought to 35% saturation with ammonium sulphate and washed with 35% saturated ammonium sulphate until the supernatant remained colourless. The pellet was then dissolved in PBS diluted 1 : 1 with distilled water (1/2 × PBS). The protein solution was dialysed extensively against 1/2 × PBS and then applied to a 250 ml column of DEAE sephadex A50 (Pharmacia) equilibrated with 1/2 × PBS. After washing out the unabsorbed proteins with 1/2 × PBS the bound material was eluted with 2 × PBS. Ten µl of pooled serum was blocked with an aliquot of the partially purified antigen equivalent to 100 ml of original culture medium. Despite the presence of large amounts of bovine IgG in the extract excellent specific inhibition of precipitation of one of the EBV antigens (143) can be seen (Fig. 4) when comparing blocked (BL) with unblocked (–) samples.

DISCUSSION

We describe an immune precipitation technique for the analysis of EBV-specified antigens. The use of a negative cell extract to preabsorb the test sera results in good quality precipitates containing few host proteins. This allows the study of sera which in addition to antiviral antibodies also contain autoantibodies. The study of a large number of sera has shown the need to prepare a pool of sera in order to standardize the results obtained in the immune precipitation test. We have also demonstrated that preabsorption of the sera with extracts containing unlabelled antigens, followed by precipitation of a standard superinfected Raji cell extract can be a useful method for the identification of antigens present in unlabelled antigen extracts. A further modification of the absorption procedure allows the identification of antigens in samples containing large amounts of IgG.

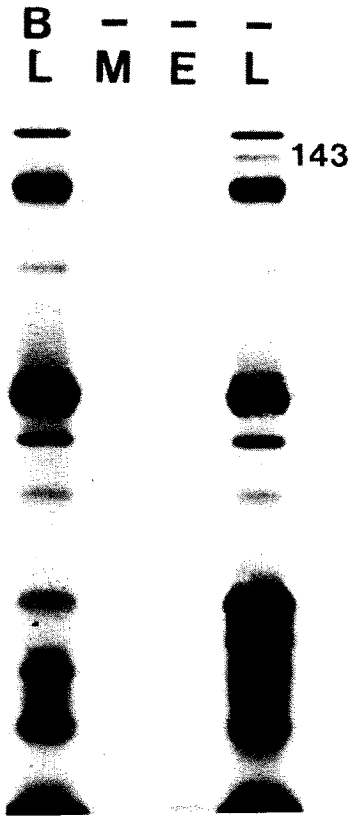

Fig. 4. Immunoprecipitation blocking assay of proteins excreted into the culture medium by P3HR1 cells. BL: serum preabsorbed with the antigen-containing fraction and used to precipitate SI Raji cells labelled from 12 to 16 h post-infection. –, Serum was not preabsorbed and precipitates were prepared from uninfected (M) and infected cells labelled from 3 to 7 (E) or 12 to 16 h post-infection.

An extract from 10^8 HRIK cells (10^7antigen-positive cells) was capable of blocking 10 µl of serum (78% of the precipitating activity was inhibited). Further quantitation is not possible at the moment. When monoclonal antibodies to the various proteins are available, the procedure could be developed further to give a quantitative instead of a qualitative assay for selected proteins in unlabelled extracts. Using the blocking test we identified at least 4 proteins (105, 90, 80, 49) in extracts of chemically induced Raji cells; these proteins are most probably members of the early or early membrane antigen complexes. Furthermore we have shown that a protein of molecular weight 143×10^3 is secreted into the culture medium by cells which are productively infected with EBV; this protein could be partially purified by a combination of ammonium sulphate precipitation and DEAE sephadex chromatography.

Using the methods described above, it should be possible to follow specific EBV

antigens through various preparative procedures without the need to carry out tedious immunofluorescent blocking assays, which to date has been the only method for the identification of EBV antigens present in extracts of unlabelled cells. The need for highly purified EBV antigens is clear; they are required for the development of specific ELISA or RIA tests which at the moment suffer from high background levels due to the non-viral antibodies present in the test sera. In addition it would be advantageous to prepare both polyclonal and monoclonal antibodies to the various antigens for use in comparative studies of different isolates of EBV and to identify proteins within an extract which are antigenically related but which have differences in their apparent molecular weights.

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