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J S Beneke; ... et. al

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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF AN INHIBITOR OF DNA SYNTHESIS PRODUCED BY AN EPSTEIN-BARR VIRUS-TRANSFORMED B CELL LINE

JANET S. BENEKE, LOUIS F. QUALTIERE, MICHAEL C. NESHEIM, AND GARY R. PEARSON

From the Departments of Microbiology and Hematology, Mayo Clinic/Foundation, Rochester, Minnesota 55901

An inhibitory factor, which has been shown to suppress the uptake of ^{125}I-iododeoxyuridine by both lymphoid and nonlymphoid cells, was isolated from the supernatant of an Epstein-Barr virus- (EBV) transformed B cell line (1605L) established from a cotton-topped marmoset. Purification of the inhibitor, which was produced in serum-free medium by crowded cultures of the 1605L cells, was achieved by DEAE-cellulose chromatography followed by preparative polyacrylamide gel electrophoresis. The apparent m.w. of the 1605L factor was determined to be 65,000 to 70,000 by SDS-polyacrylamide gel electrophoresis. The inhibitor was sensitive to digestion by trypsin and chymotrypsin but not RNase or DNase, indicating that it was protein in nature. Exposure of the 1605L factor to 56°C for ½ hr or to pH 2 for 48 hr at 4°C destroyed its inhibitory activity. The biochemical characteristics and activity of the 1605L inhibitor distinguish it from Type I interferon and several other soluble immunologic mediators known to be produced by lymphoid cell lines.

Continuous lymphoblastoid cell lines have been observed to produce a number of soluble mediators of immunologic significance, including lymphotoxin (1), migration inhibitory factor (2), immunoglobulins (3), and interferon (4). In addition, a number of factors that suppress the mitogen responsiveness of lymphocytes, and that appear to be distinct from the factors listed above, have been detected in the supernatants and cell extracts of lymphoblastoid cell lines. Although several of these antiproliferative factors have been characterized extensively, none has so far been purified to homogeneity, and detailed physicochemical description of these molecules has, therefore, not been possible.

In the accompanying paper, the activity of soluble inhibitors of DNA synthesis detected in the cellfree supernatant culture fluids of *Herpesvirus saimiri* (HVS)-[1] and Epstein-Barr virus- (EBV) transformed lymphoid cell lines were described. These inhibitory factors produced by the two cell lines, which were established from cotton-topped marmosets with EBV- or HVS-induced lymphomas, are of particular interest, since the inhibitors may play a role not only in the normal regulation of the immune response but in the expression of malignant disease induced by these viruses as well. In this report, we describe the isolation and biochemical characterization of the inhibitor produced by the EBV-transformed B cell line (1605L).

MATERIALS AND METHODS

Cell culture and production of the inhibitor. The 1605L cell line was established from a lymphosarcoma nodule in the liver of a cotton-topped marmoset after experimental infection with EBV (5) and was obtained through the courtesy of Dr. Harvey Rabin, Frederick Cancer Research Center, Fort Detrick, Frederick, Maryland. The cells were free of detectable levels of mycoplasma contamination. The cell line was maintained on Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin. The inhibitor was produced by concentrated cell cultures in serum-free medium as described previously (6).

Blastogenesis assay system. The PHA blastogenesis test employing baboon lymphocytes that was described in detail in the accompanying paper (6) was used as the assay system in the purification and characterization of the 1605L inhibitor. The activity units in an inhibitory sample tested in this assay were defined as the reciprocal of the dilution of the sample, which gave a 50% reduction in the uptake of ^{125}I-iododeoxyuridine (^{125}I-IUDR) by the PHA-stimulated lymphocytes in comparison with control cultures that received no inhibitor.

DEAE-cellulose chromatography. DEAE-cellulose (Cellex D, Bio-Rad, Richmond, Calif.) was equilibrated with 0.01 M phosphate buffer (pH 8.0). Serum-free inhibitory supernatants that had been extensively dialyzed against 0.01 M phosphate buffer (pH 8.0) were applied to columns packed with DEAE-cellulose. After the unbound protein had been washed from the column with the phosphate buffer, a linear gradient of 0 to 0.4 M NaCl in 300 ml of 0.01 M phosphate buffer was passed over the column, eluting the protein that had bound. Fractions were assayed for their protein content by absorbance at 280 nm or by the method of Lowry *et al.* (7). When protein was assayed by absorbance at 280 nm, one absorbance unit was defined as being equal to a concentration of protein of 1 mg/ml. Column fractions were monitored for suppressor activity in the baboon lymphocyte blastogenesis assay.

Preparative polyacrylamide-gel electrophoresis. The apparatus and procedure used for the preparative polyacrylamide-gel electrophoresis have been described in detail by Nesheim (8). To prepare the gel apparatus for electrophoresis, a gel solution composed of 7% (w/v) acrylamide, 0.029% TEMED (see Abbreviations), and 0.07% ammonium persulfate in a buffer of 50 mM Tris and 50 mM boric acid (pH 8.3) was poured into

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[1] Abbreviations used in this paper: HVS, *Herpesvirus saimiri*; EBV, Epstein-Barr virus; ^{125}I-IUDR, ^{125}I-iododeoxyuridine; PBS, Dulbecco's phosphate-buffered saline; TEMED, *N,N,N',N'*-tetramethylethylenediamine; SDS, sodium dodecylsulfate.

the cylindrical chamber of the apparatus to a height of 5 cm and overlayed with H_2O. After polymerization, the electrode buffer, also of 50 mM Tris and 50 mM boric acid, was added to reservoirs above and below the gel. The gel was pre-electrophoresed for 1 hr at 100 volts. Then 20 mg ascorbic acid in 2 ml 0.025 M NaOH was made with 10% glycerol, bromphenol blue dye was added, and this sample was electrophoresed until the dye front, which included the ascorbic acid, was eluted from the gel. Fractions containing inhibitory activity obtained from the DEAE-cellulose column were prepared for electrophoresis by concentrating to a volume of less than 5 ml by ultrafiltration through an immersible molecular separator with a 10,000 m.w. cut-off (Millipore Corp., Bedford, Mass.), dialyzing the sample against the Tris-borate buffer, then adding bromphenol blue dye and 10% glycerol. The sample was layered on top of the gel, and electrophoresis was begun at 50 volts for the first hr and 100 volts thereafter. Sample eluted from the gel was collected in fractions that were assayed for inhibitory activity in the baboon lymphocyte blastogenesis assay. Starting samples containing from 2 to 14 mg of protein were successfully separated by this technique.

Analytical polyacrylamide-gel electrophoresis. Active fractions from each isolation step were analyzed for purity by polyacrylamide-gel electrophoresis according to the discontinuous method of Ornstein (9) and Davis (10). After electrophoresis, gels were stained with 1% amido black in 7% acetic acid and destained in 7% acetic acid, or were stained with a solution of 0.25% Coomassie Brilliant Blue, 10% acetic acid, and 50% methanol and destained in a solution of 5% methanol and 7% acetic acid.

For m.w. determination, samples were analyzed by the discontinuous, sodium dodecylsulfate- (SDS) gel electrophoresis system of Laemmli (11). Samples were reduced before electrophoresis by boiling for 2 min in the presence of 10% 2-mercaptoethanol and 2% SDS. After electrophoresis, gels were stained with Coomassie Blue and destained as described previously. The following standard proteins were used in determining m.w.: human serum albumin, 68,000; ovalbumin, 43,000; soybean trypsin inhibitor, 21,000; and lysozyme, 12,000 (all obtained from Pharmacia, Piscataway, N. J.).

Recovery of inhibitory activity from analytical polyacrylamide gels. To determine the electrophoretic mobility of the component with inhibitory activity in the active fraction after preparative gel electrophoresis, the same sample was electrophoresed on identical cylindrical (5 mm x 10 cm) Ornstein-Davis gels of 10.5% polyacrylamide. One gel was stained with Coomassie Blue, whereas the remaining gel was sliced into 2-mm slices by a Gilson automatic gel fractionator (model B-100/GMA-GCB). Two milliliters of Dulbecco's phosphate-buffered saline (PBS) were added to each macerated gel fraction, and then the fractions were incubated for 48 hr at 4°C to allow the protein to elute from the gel. Samples were then individually filtered through 0.45-μ Swinnex filters (Millipore Corp.) to remove gel particles and dialyzed overnight against PBS to remove low m.w. substances that were toxic to lymphocytes. Fractions were then tested for activity in the baboon lymphocyte blastogenesis assay in order to establish the electrophoretic mobility of the activity. The mobility of the activity was then compared with the mobility of the protein components found in the stained gel.

Enzyme digestion experiments. The chemical nature of the 1605L inhibitor that had been purified by preparative gel electrophoresis was determined by its sensitivity to digestion by various enzymes. Ribonuclease-A (type X11-A containing 88 Kunitz units/mg; Sigma, St. Louis, Mo.) and deoxyribonuclease I (containing 2040 Kunitz units/mg protein; Sigma) were each added at a concentration of 100 μg/ml to aliquots of the 1605L inhibitor diluted in PBS containing approximately 2.5 μg/ml protein. Samples were incubated for 1 hr at 37°C and then dialyzed against PBS overnight at 4°C to remove low m.w. products of the nucleic acid digestion that would interfere in the subsequent DNA synthesis assay.

To test the inhibitor for sensitivity to trypsin, an aliquot diluted to 2.5 μg/ml in PBS was treated with bovine pancreatic trypsin Type I (Sigma) at a concentration of 1.5 mg/ml containing approximately 16,050 units/ml. After 1 hr incubation at 37°C, 1.5 mg/ml soybean trypsin inhibitor (Type 1-5; Sigma) were added, and the sample was stored at 4°C until assayed.

Determination of temperature and pH sensitivities of the inhibitor. Samples of the 1605L inhibitor that were purified by preparative gel electrophoresis were tested for sensitivity to exposure to heat. The samples, which were diluted in PBS, were placed in 37°C or 56°C water baths for various periods of time before being assayed for activity against PHA-stimulated baboon lymphocytes.

To test the sensitivity of the 1605L inhibitor activity to low pH conditions, an aliquot of the purified inhibitor that had been diluted in PBS was lowered to pH 2 by the addition of 1 M HCl. The sample was held at pH 2 for 48 hr at 4°C before being titrated back to pH 7.2 with 1 M NaOH. The inhibitory activity in the sample was then tested in the PHA-blastogenesis assay by using a sample of PBS that had been similarly diluted and pH adjusted as control.

RESULTS

Purification of the factor. Ion-exchange chromatography with DEAE-cellulose was found to be useful as an initial purification step for the 1605L inhibitor (Fig. 1). The crude, serum-free supernatant containing the inhibitor was applied to the column in the 0.01 M phosphate starting buffer, and protein that was not bound to the column was eluted in the flow-through fraction. Then a linear gradient of NaCl from 0 to 0.4 M was used to elute protein that had been bound to the column. When the fractions were tested for activity, all of the activity was found to be present in a discrete peak that was eluted in the NaCl gradient at approximately 0.15 M NaCl, and none of the activity was found in the flow-through fraction. About 75% of the total protein and 75% of the activity units applied to the column could routinely be recovered after chromatography, and the specific activity defined as activity units/mg protein of the active fraction obtained by this technique was typically increased 8- to 10-fold over the crude starting material.

Because of the relatively rapid mobility and apparently good resolution of the active component on the analytical Ornstein-Davis gels, preparative polyacrylamide gel electrophoresis was evaluated as a second purification procedure to follow DEAE-cellulose chromatography. A continuous gel system employing Tris-borate buffer that gave good resolution of the active component and yields of total protein and activity of around 60% was selected as the standard preparative gel technique for purifying the 1605L inhibitor. Figure 2 shows the A280 and activity profile of fractions obtained from the preparative gel. Although the absorbance readings of these fractions were always low due to the extensive dilution of the protein with the elution buffer, the pattern shown in Figure 2 was found to be highly reproducible in 10 separate experiments. In addition, activity was consistently found to be eluted from the prepara-

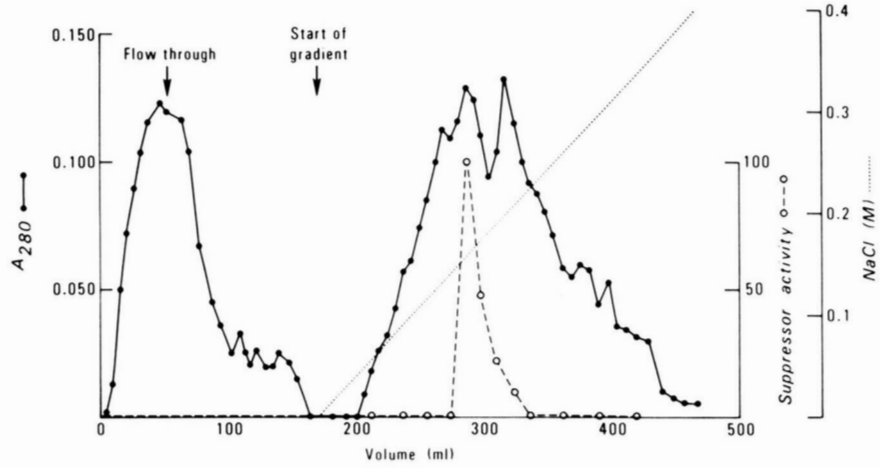

Figure 1. DEAE-cellulose chromatography of the crude 1605L inhibitor. Crude 1605L serum-free supernatants containing approximately 34 mg protein were dialyzed extensively against 0.01 M phosphate buffer (pH 8.0). The sample (15 ml) was applied to the column (2.5 x 20 cm) that had been equilibrated in the same buffer. Column fractions were monitored for suppressor activity in the baboon lymphocyte blastogenesis assay.

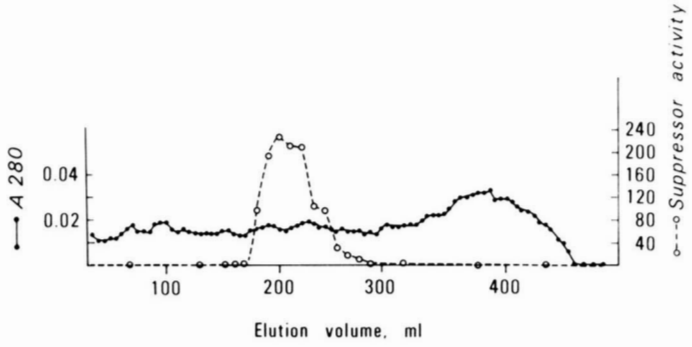

Figure 2. Preparative gel electrophoresis of the 1605L inhibitor. Inhibitory fractions from the DEAE-cellulose column were concentrated to 5 ml, then dialyzed against 50 mM Tris-borate buffer (pH 8.3). A 5-cm polyacrylamide gel in the same buffer was used to separate the components of the sample. Electrophoresis was conducted at 50 volts for the first ½ hr and at 100 volts thereafter. Material eluting from the gel was transported with 50 mM Tris-borate buffer at a flow rate of 1.7 ml/min to a fraction collector. Suppressor activity of the fractions was determined in the lymphocyte blastogenesis assay.

tive gel in one peak in the position shown in Figure 2. The specific activity of the active fraction obtained after preparative gel electrophoresis was increased 8- to 10-fold over the fraction obtained from the ion-exchange column. The overall enrichment in terms of specific activity of the active fraction from the preparative gel, over the crude starting material, was calculated to be around 70-fold, with a recovery of activity of 26%. Inhibitory activity could be detected in samples containing as little as 25- to 50 ng/ml protein.

When the active fraction obtained from the preparative gel was analyzed on Ornstein-Davis polyacrylamide gels, a major component and at least one minor component were identified (Fig. 3). To distinguish the component with which activity was associated, aliquots of the same active fraction obtained from the preparative gel were electrophoresed on duplicate Ornstein-Davis gels of 10.5% polyacrylamide. One gel was stained with Coomassie Blue dye and subsequently scanned, whereas the other gel was sliced into 2-mm slices, the protein was eluted from the slices, and the eluates were tested for activity. The activity was found to correspond with the major protein component in the fraction (Fig. 4).

The apparent m.w. of the 1605L inhibitor was determined by SDS-polyacrylamide gel electrophoresis. A sample purified by preparative gel electrophoresis was reduced and then analyzed on discontinuous SDS-polyacrylamide gels. Comparison of the relative mobility of the major component containing the inhibitory activity with the standards of known m.w. revealed that

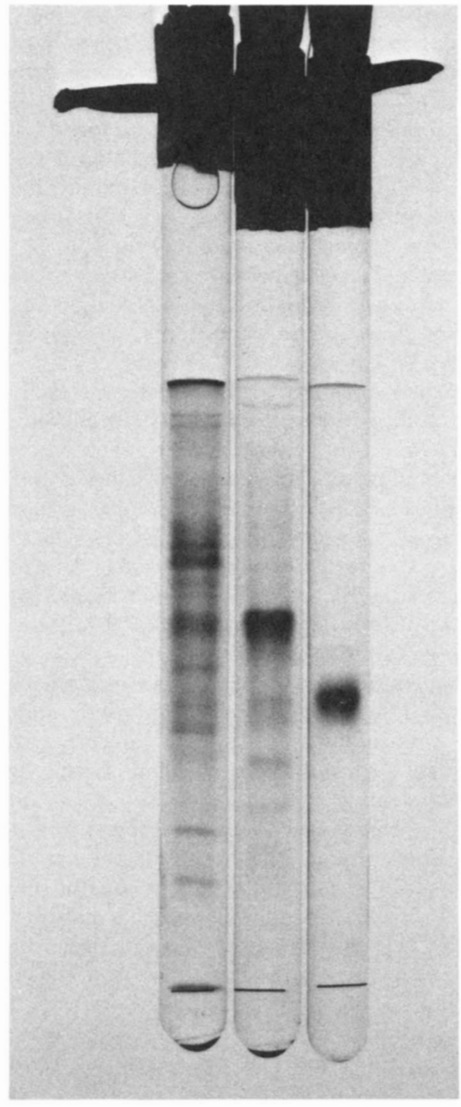

Figure 3. Analytical polyacrylamide gel electrophoresis of the 1605L inhibitor after purification steps. *Left,* crude 1605L inhibitor; *middle,* inhibitor after DEAE-cellulose chromatography; *right,* inhibitor after preparative gel electrophoresis. Electrophoresis was done according to the technique of Ornstein and Davis (9, 10).

the 1605L inhibitor had an apparent m.w. of 65,000 (Fig. 5). Similar experiments (not shown) have confirmed that the apparent m.w. of the inhibitor is in the 65,000 to 70,000 range.

Characterization of the inhibitor. Enzyme digestion experi-

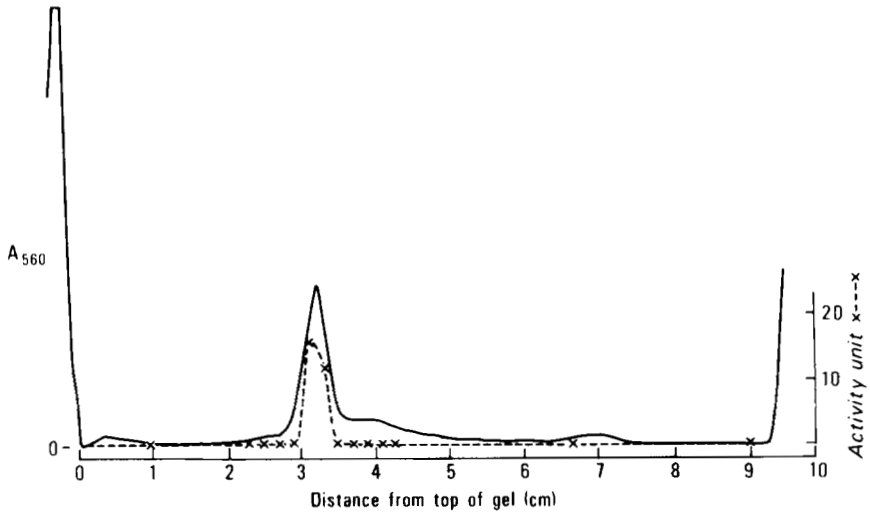

Figure 4. Correspondence of the 1605L inhibitor activity with the major protein component after preparative gel electrophoresis. Samples of the 1605L inhibitor purified by preparative gel electrophoresis were run on duplicate Ornstein-Davis gels. One gel was stained and scanned, whereas the other was sliced, and eluates from the slices were tested for activity in the lymphocyte blastogenesis assay. Activity is expressed as units/milliliter of eluate.

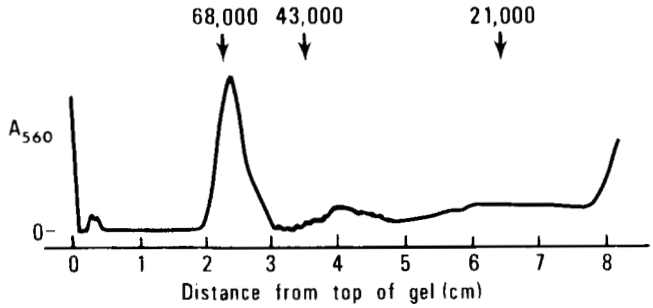

Figure 5. Determination of apparent m.w. of the 1605L inhibitor by SDS-polyacrylamide gel electrophoresis. A sample of the 1605L inhibitor that had been purified by preparative gel electrophoresis was reduced with 2-mercaptoethanol and electrophoresed according to the method of Laemmli (11). The m.w. of the 1605L inhibitor, the major component in this sample, was determined by comparison of its electrophoretic mobility with that of known m.w. standards, indicated by arrows.

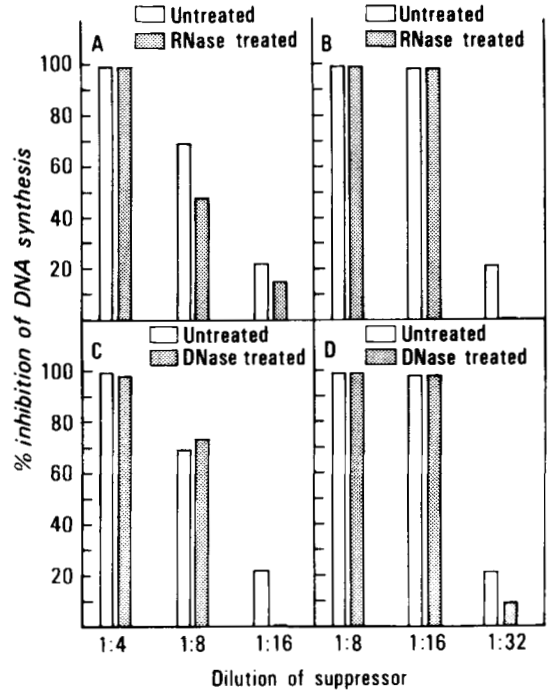

Figure 6. Effects of RNase and DNase digestion on the activity of the 1605L inhibitor. The inhibitor was tested for activity against PHA-stimulated baboon lymphocytes. The results of two separate experiments employing different preparations of the factor are shown. *A* and *B, unshaded bars*, untreated 1605L inhibitor; *shaded bars*, RNase-treated 1605L inhibitor; *C and D, unshaded bars*, untreated 1605L inhibitor; *shaded bars*, DNase-treated inhibitor.

ments were performed in order to determine the probable chemical nature of the 1605L inhibitor. In these experiments, portions of the purified inhibitor were treated with enzymes in the absence of serum supplements before being diluted and tested in the lymphocyte blastogenesis assay along with untreated inhibitor and controls that contained enzymes but no inhibitor. The results of two such experiments in which two different preparations of the 1605L inhibitor were treated with RNase or DNase are shown in Figure 6. The activity of the 1605L inhibitor was not significantly diminished by exposure to either RNase or DNase in these experiments. The digestion of the inhibitor with trypsin, however, consistently reduced its activity in the lymphocyte blastogenesis assay, as is evidenced by the data from three similar experiments shown in Figure 7 (panels A, B, and C). This was most evident with the 1:8 dilution of inhibitor in panel A, the 1:16 and 1:32 dilutions in panel B, and the 1:32 dilution in panel C. All of these reductions were significant at $p < 0.001$. The fact that the loss of activity was most apparent with the higher dilutions of the inhibitor suggest that the trypsin treatment had to reduce the inhibitor concentration below a certain threshold level before obvious loss of activity was apparent. The 1605L inhibitor was also sensitive to digestion by chymotrypsin (data not shown).

In other experiments in which the activity of the 1605L factor was tested after exposure to extreme temperature and pH conditions, the protein was found to be sensitive to heating at 56°C for ½ hr, unaffected by heating at 37°C for 1 hr, and sensitive to exposure to pH 2 for 48 hr at 4°C.

DISCUSSION

An inhibitor of DNA synthesis, produced by an EBV-transformed B cell line, was extensively purified by DEAE-cellulose chromatography and subsequent preparative gel electrophoresis. Experiments performed on the purified inhibitor indicated that it was a trypsin- and chymotrypsin-sensitive protein that was labile at 56°C for ½ hr and destroyed by exposure to pH 2. The apparent m.w. of the factor was determined by SDS-gel electrophoresis to be 65,000 to 70,000. Comparison of the banding pattern obtained after gel electrophoresis under reduced

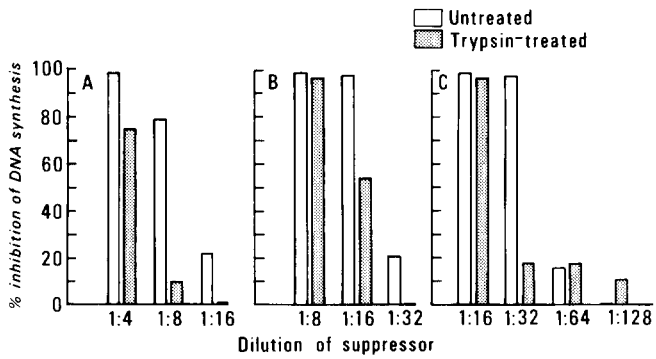

Figure 7. Effects of trypsin digestion on the activity of the 1605L inhibitor. The inhibitor was tested for activity against PHA-stimulated baboon lymphocytes. The results of three separate experiments with different preparations of the factor are shown. *A*, *B*, and *C*, *unshaded bars*, untreated 1605L inhibitor; *shaded bars*, trypsin-treated 1605L inhibitor. The percent inhibition of DNA synthesis caused by the trypsin-treated 1605L inhibitor was calculated by using control values obtained in stimulated lymphocyte cultures exposed to the same concentration of trypsin and trypsin inhibitor in the absence of the 1605L inhibitor.

and nonreduced conditions (data not shown) suggested that the factor consisted of a single polypeptide chain.

The characteristics that have been determined for the 1605L inhibitor distinguish it from lymphokines that have immunosuppressive potential, such as MIF and LT. The 1605L inhibitor can also be distinguished from interferon on the basis of its activity and physical characteristics. Type I interferon has been reported to be resistant to exposure to pH 2 (12), and to have components of apparent m.w. 15,000 and 19,000 to 21,000 (13, 14), in contrast to the 1605L inhibitor, which was found to be sensitive to pH 2 and to have an apparent m.w. of 65,000 to 70,000. The properties of the 1605L inhibitor also distinguish it from the inhibitor associated with mycoplasma contamination, which was reported to have a m.w. of 78,000 (15) and was resistant to trypsin (16, 17) and to heat (16, 18).

In terms of activity and the physical data available, the 1605L factor appears to be very similar to many of the inhibitors that have been described that are produced by other cell lines, none of which have been purified. It is of particular interest that the 1605L inhibitor is similar to the factor described by Neubauer *et al.* (19), since this factor was produced by an HVS-transformed marmoset T cell line. The factor described in this and the preceding paper (6) that is produced by an EBV-transformed marmoset B cell line, and the inhibitor reported by Neubauer *et al.* (19, 20) both inhibited DNA synthesis of mitogen-stimulated lymphocytes and lymphoid cell lines, and neither demonstrated tissue specificity or species specificity against the target cells that they affected. The anti-proliferative factor from the marmoset T cell line described by Neubauer *et al.* (19) appeared to be a protein with heat and pH sensitivities similar to the factor purified from the 1605L cell line. However, Neubauer *et al.* (19) reported that their inhibitor was resistant to trypsin digestion, whereas the 1605L inhibitory activity was reduced by this enzyme. The m.w. range of 20,000 to 70,000 that was reported for the T cell factor (21) does not allow differentiation of the factors on the basis of m.w.

The available information also suggests that the 1605L inhibitor could be identical to the inhibitor described by Lightbody *et al.* (22), which was produced by a human EBV-transformed B cell line. The Lightbody factor, which was nondialyzable, inhibited the mitogen responsiveness of lymphocytes and was not species specific. An inhibitory factor produced by crowded human lymphoblastoid cell lines that may also have been EBV-transformed B cell lines was characterized by Green *et al.* (23) and Green and Wistar (24). It was similar to the 1605L factor, in that it was a protein of 40,000 to 70,000 m.w. that was not species specific in its activity. However, the authors reported that this factor, in contrast to the 1605L inhibitor, did not suppress DNA synthesis of established lymphoid cell lines. Recently, Vesole *et al.* (25) have detected inhibitors of PHA blastogenesis in the supernatants of both B and T cell lines. These factors were estimated by gel filtration chromatography to have m.w. of 70,000, which is consistent with the m.w. value that has been obtained by the 1605L inhibitor.

The significance of the inhibitors of DNA synthesis produced by lymphoid cell lines as regulators of the immune response *in vivo* has not yet been established. The possible role of these inhibitors in the expression of malignant disease is of particular interest. In patients with the EBV-associated disease, African Burkitt's lymphoma, reduced delayed hypersensitivity responses to a battery of antigens were reported to be correlated with the tumor burden (26, 27). In addition to the impaired cutaneous reactivity, the lymphocyte responses to PHA and percentages of T cells in the peripheral blood of these patients were also depressed (28). The development of lymphoma in owl monkeys after experimental infection with HVS was also shown to be accompanied by a marked depression in the lymphocyte responses to mitogens (29, 30). Furthermore, the nonreactive lymphocytes (30) and plasma (20) from diseased monkeys conferred loss of PHA reactivity to normal lymphocytes. It is possible that the lymphoid tumor cells in patients with EBV-induced lymphoma and the transformed cells in the experimental animals with HVS-induced disease may be relasing a factor that suppresses the host's immune system and may thereby favor the proliferation of the tumor cells. The inhibitor could be specifically a product of the virally transformed cells, or it could be a normal regulator of the immune response that is inappropriately produced by the tumor cells. Work is now underway in our laboratory to develop an antiserum that would be specific for the purified 1605L inhibitor. Such an antiserum will be a useful tool in experiments designed to further characterize the mechanism of action of this factor and to assess its role in the malignant disease process.

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