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SPECIFIC ENHANCEMENT OF IMMUNE RESPONSES BY BCG: ISOLATION OF EXTRACELLULAR DNA FROM SUPERNATANTS OF SPECIFICALLY STIMULATED BCG-PRIMED LYMPHOID CELLS[1]

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When lymphocytes obtained from W/Fu rats primed with BCG are cultured in the presence of PPD, they elaborate a factor that is capable of potentiating the specific *in vitro* generation of cytotoxic lymphocytes to syngeneic (C58NT)D lymphoma cells and to BN alloantigen.

Purification of the factor, achieved by gel filtration on Sephadex G-100, was facilitated by using a serum-free culture condition and the removal of the specific stimulating antigen, PPD, after an initial incubation period. The factor isolated contains DNA by its absorption spectrum, resistance to trypsin and RNAase, but complete susceptibility to DNAase, and by the presence of ethidium bromide-positive material in the purified sample. It displays a 260/280 nm absorption ratio of 1.6 and a m.w. estimate of 10,000 to 30,000. Electrophoresis of the purified factor on agarose gel yielded three ethidium bromide reactive bands. Data obtained following the slicing and elution of these bands, and then testing for potentiating activity indicated that two of the three bands contained potentiating activity.

In an earlier report (1) we showed that the addition of lymphoid cells, obtained from W/Fu rats previously immunized with bacillus Calmette-Guérin (BCG),[2] to cultures of lymphoid cells responding to a Gross virus-induced lymphoma, (C58NT)D, potentiated the generation of cytotoxic effector cells specific only against (C58NT)D tumor cells and not to an unrelated BN lymphoma. The addition of purified protein derivative (PPD) to these cultures was absolutely necessary for the potentiating effect of BCG-primed cells. The efficacy of these highly cytotoxic effector cells was also tested *in vivo* by using the Winn assay. When the effector cells generated in this manner were mixed with (C58NT)D tumor cells and injected subcutaneously in a Winn assay, significantly greater inhibition of tumor growth was observed compared with cells cultured without BCG-primed cells and PPD (1). A similar potentiating effect on the generation of cytotoxic effector cells was observed when the supernatant of PPD-stimulated BCG-primed cultures was added to (C58NT)D-primed responding cells or to a one-way mixed leukocyte culture (2). These observations indicate that a soluble product of BCG-primed lymphoid cells can increase the reactivity of an independent lymphoid cell population responding to tumor antigens or alloantigen. The kinetics of release of the immune potentiating factor, IPF, showed that it started to be elaborated into culture by 2 hr and peaked at 6 to 12 hr (2). Supernatants obtained after 30 hr of incubation showed no potentiation; however, heating this supernatant to 56°C for 40 min reinstated its potentiating activity. These findings suggested that the apparent loss of potentiating activity in culture supernatants after 30 hr of incubation was due probably to the presence of a heat-labile inhibitor, whereas the factor itself was heat stable. In the present report, the partial purification of the potentiating factor was achieved and shown to contain DNA.

Two important modifications were introduced in the culture conditions used in the present study that expedited the isolation of the IPF. The culture medium was serum-free, and the BCG-primed cells were washed free of PPD before a second incubation from which the supernatants used in this study were obtained.

MATERIALS AND METHODS

Animals. Inbred male Wistar Furth (W/Fu) rats were obtained from Microbiological Associates, Walkersville, Md.

Tumor. The Gross virus-induced W/Fu rat lymphoma (C58NT)D was kindly supplied to us by Dr. Ronald Herberman, National Cancer Institute, Bethesda, Maryland. The tumor was maintained by passage of 20 to 25 × 10^6 cells in ascitic form in weanling W/Fu rats. The (C58NT)D tumor will grow transiently and then regress when inoculated at a dose of 10^6 to 10^8 cells subcutaneously into 10- to 12-week-old male rats.

Primary immunizations with tumor cells and BCG, secondary *in vitro* restimulation, determination of proliferative activity, and cell-mediated cytotoxicity have been described in detail in previous reports (1, 3, 4).

Preparation of cellfree culture supernatant: Cell cultures were prepared from spleens of BCG-immune W/Fu rats 4 to 6 weeks after subcutaneous injection with 10^8 viable organisms of Phipps strain BCG (Trudeau Institute, Saranac Lake N. Y.). Adherent cell populations were removed on plastic plates as previously described (2). The nonadherent cell population was suspended in 40 ml of serum-free enriched high amino acid (EHAA) medium (5) at 2.6 × 10^6 cells/ml with and without PPD (10 μg/ml, Connaught Laboratories, Willowdale, Canada).

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[1] This work was supported by Grants CA-19170, CA-19148, and CA-19149 from the National Cancer Institute and was performed while the author was in the Division of Tumor Immunology, Fred Hutchinson Cancer Research Center, Seattle, Washington.
[2] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; PPD, purified protein derivative; IPF, immune potentiating factor; EHAA, enriched high amino acid; EDTA, ethylenediaminetetraacetic acid.

After 6 hr incubation at 37°C in air containing 5% CO_2, the cultures were centrifuged at 2000 × G for 15 min. To remove PPD, the cells were washed four times in Eagle's medium and then resuspended in 40 ml of fresh serum-free EHAA medium for 16 hr at 37°C. This supernatant was dialyzed for 48 hr against phosphate-buffered saline (PBS) and concentrated 100-fold by using Amicon ultrafiltration PM-10 membrane (nominal retention of m.w. 10,000). Since this supernatant contained no detectable amount of PPD, it was used for further purification. In testing the concentrated supernatant for possible contamination by PPD, 0.1-ml aliquots were injected intradermally into left flanks and 0.1 μg PPD into right flanks of BCG-primed W/Fu rats. Twenty-four to 48 hr later, the animals were examined for delayed hypersensitivity. Although the side injected with PPD showed positive reaction averaging 12.5 ± 1.3 mm, no reaction could be seen on the side tested with concentrated supernatant. The A_{280} of the sample tested was 1.6, and this test would easily detect 0.01 μg of PPD.

Enzymatic treatment of purified IPF. Polyacrylamide trypsin, agarose-RNAase (Sigma Chemical, St. Louis, Mo.), and Sepharose-DNAase (Worthington Biochemical Co., Freehold, N. J.) were suspended and washed in PBS. Equal amounts of purified IPF were tested for sensitivity to trypsin and RNAase digestion in PBS. DNAase digestion was carried out in PBS containing 5 mM $MgCl_2$ and $CaCl_2$. The digestions were allowed to proceed for 5 hr at 37°C on a rotary shaker, after which the supernatants were removed and dialyzed against PBS. The three samples and undigested aliquot were added to cultures of (C58NT)D-responding spleen cells and mitomycin C-inactivated (C58NT)D tumor cells for 5 days. Cytotoxicity was then determined by using the standard ^{51}Cr-release assay.

Gel electrophoresis. Agarose slab gel, 0.7% was prepared in Tris-acetate buffer (90 mM Tris-OH, 90 mM sodium acetate, 40 mM EDTA[2]), adjusted to pH 8 with acetic acid. Samples were applied in 50 μl of Tris-acetate buffer containing sucrose (20% w/v). Electrophoresis was at 35 volts for 8 hr. The DNA band was stained with ethidium bromide and visualized by using a short wavelength ultraviolet light. The fluorescent bands were photographed by using a Vivitar orange (02) filter and Polaroid paper.

Column calibration. To estimate the m.w. of the potentiating activity, the column was calibrated by using rabbit muscle phosphorylase b (97,000 m.w.), bovine serum albumin (68,000 m.w.), rabbit muscle troponin C (17,500 m.w.), and dogfish parvalbumin (12,500 m.w.). The relative migration of these protein markers was compared with that of the potentiating molecule, and from this an apparent m.w. of 12,000 to 14,000 was estimated.

RESULTS

Production of immune potentiating factor by BCG-primed nonadherent lymphocytes. Nonadherent lymphoid cells were obtained from BCG-primed or normal W/Fu rats by fractionating the splenic cells on plastic dishes. In initial experiments, the nonadherent cells were incubated alone, as control, or with PPD (10 μg/ml) for 16 hr at 37°C. The cellfree supernatants obtained after this incubation period were used to directly culture (C58NT)D-immune or normal W/Fu lymphocytes and inactivated (C58NT)D tumor cells. For control, (C58NT)D-immune lymphocytes were cultured with mitomycin C-inactivated syngeneic thymocytes. After a 5-day culture period, the effector cells generated were tested in a 4-hr ^{51}Cr-release assay by using as targets (C58NT)D tumor cells. The results obtained are summarized in Table I. (C58NT)D-immune lymphocytes cultured with tumor cells generated good specific cytotoxic response against the tumor target cells. Effector cells generated in the culture supernatant of BCG-primed cells and PPD (C) were, however, significantly more cytotoxic to tumor cells compared with cells cultured in nonconditioned medium (E). No significant cytotoxicity was observed when cells from normal W/Fu rats were incubated with tumor cells either in EHAA medium alone (B) or conditioned medium from BCG-primed cells and PPD (A). Most significantly, without added PPD the supernatant from BCG-immune cells showed no potentiating effect (D); likewise, the addition of PPD alone to cultures of (C58NT)D-primed cells and (C58NT)D tumor cells resulted in no augmentation of cytotoxicity (E). These results indicated that soluble IPF is elaborated by BCG-primed, nonadherent, lymphoid cells only when stimulated with PPD.

Next, it was determined whether the PPD was essential to be present in the supernatant with the IPF in order to observe potentiation. To accomplish this, BCG-primed cells were incubated with PPD for 6 hr, after which the cells were centrifuged and washed four times with 50 ml each of Eagle's medium. The cells were then resuspended in EHAA medium for 16 hr at 37°C. When the first 6-hr supernatant containing PPD and the 16-hr supernatant without PPD were tested for their potentiating effect, both showed very good potentiation (Fig. 1). This data suggests that the IPF can function by itself, without complexing with PPD, to potentiate the generation of cytotoxic effector cells. It also suggests that the stimulation of BCG-primed cells by PPD required the stimulant to be present for only a brief period (6 hr), after which its presence was no longer important, despite the continued elaboration of the factor for at least another 16 hr.

Gel filtration chromatography of the immune potentiating supernatant. Immune potentiating supernatant obtained after removing PPD was dialyzed for 24 hr and concentrated 100-fold by using Amicon PM-10 membrane. In preliminary experiments, ultrafiltration through a PM-10 membrane retained all of the potentiating activity, whereas after filtration through PM-30 membrane, all activity was recovered in the effluent. This suggests that the m.w. of the factor was not higher than

TABLE I

Production and effect of immune potentiating factor

Nonadherent Spleen Cells	PPD (10 μg/ml)	(C58NT)D_{im} + (C58NT)D_m[a]			(C58NT)D_{im} + Thy_m[b]			Normal Spleen + (C58NT)D_m[c]		
		% Cytotoxicity[d]								
		100:1	30:1	10:1	100:1	30:1	10:1	100:1	30:1	10:1
A. Normal W/Fu	+	65	54	28	12	10	6	10	9	10
B. Normal W/Fu	−	68	51	31	10	10	8	11	9	10
C. BCG-primed W/Fu	+	96	82	56[e]	10	12	9	10	12	8
D. BCG-primed W/Fu	−	64	47	23	8	6	8	11	11	9
E. None	+	60	50	26	10	12	8	12	10	8

[a] (C58NT)D immune spleen cells + mitomycin C-inactivated (C58NT)D tumor cells.

[b] (C58NT)D immune spleen cells + mitomycin C-inactivated syngeneic thymocytes.

[c] Normal W/Fu spleen cells + mitomycin C-inactivated (C58NT)D tumor cells.

[d] Specific cytotoxicity = *a* to *b*.

[e] Statistical significance of C as compared to E at E/T of 30:1 was $p < 0.001$.

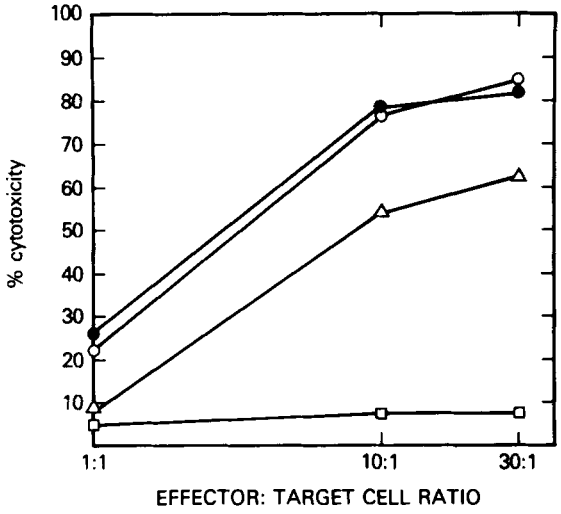

Figure 1. The effect of IPF on the generation of CTL in the presence (○) or after removal (●) of PPD as compared to medium alone (△) ($p < 0.01$). The control (□) consists of (C58NT)D immune lymphocytes cultured with syngeneic thymocytes in the presence or absence of IPF.

30,000. The PM-10 concentrated supernatant was applied to a Sephadex G-100 column (1.5 x 100 cm), equilibrated with PBS, and developed by using the same buffer. Aliquots of 1.0 ml or 2.0 ml from every fourth fraction were filtered-sterilized and tested for potentiating activity in the following manner: 60×10^6 (C58NT)D-immune cells, or as control, syngeneic thymocytes, were co-cultured with 2×10^6 inactivated tumor cells for 5 days in serum-free EHAA medium or medium plus the different chromatographic fractions. The result of a typical experiment is illustrated in Figure 2. Some potentiating activity emerged with the column void volume, but higher specific activity fractions eluted at position corresponding to m.w. 12,000 to 14,000. These fractions (28 through 40) were pooled and concentrated 40-fold.

Dose-dependent effect of IPF. The effect of varying concentrations of the IPF was determined by limiting dilutions. The active fractions obtained from the Sephadex G-100 column was concentrated, and different amounts were added to cultures of (C58NT)D-primed cells and inactivated tumor cells. The results illustrated in Figure 3 indicate that although dilutions of 1/80 to 1/400 caused enhancement of development of cytotoxic effector cells, higher concentrations were suppressive. The exact nature of this inhibitory effect is not at present clear. Cell viability in the suppressed cultures was not significantly different from cultures that showed potentiation.

Effect of mitomycin C and puromycin on the production of IPF. The effects of mitomycin C, inhibitor of DNA synthesis, and puromycin, inhibitor of protein synthesis, on the elaboration of the IPF were investigated. Nonadherent spleen cells from BCG-immune W/Fu rats were treated with 50 μg/ml mitomycin C or 60 μg/ml puromycin for 1 hr at 37°C and then washed three times in Eagle's medium. After each wash the cells were incubated for 10 min at 37°C, and finally 2.6×10^6 cells/ml were incubated with 10 μg/ml PPD for 16 hr. The cellfree supernatants obtained were used to culture (C58NT)D immune lymphocytes and tumor cells. The results summarized in Table II indicate that both mitomycin C and puromycin, at the concentrations used, inhibited the elaboration of the factor.

Characterization of IPF. The biochemical identity of the IPF was investigated by subjecting it to digestion with immobilized DNAase, RNAase, and trypsin. The active fraction

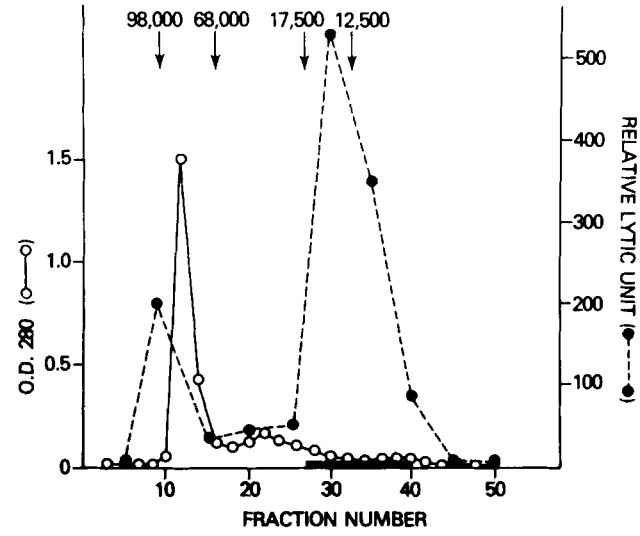

Figure 2. Gel filtration of the concentrated cellfree supernatant on Sephadex G-100 column (15. × 100 cm). Protein was determined at 280 nm (○——○) and potentiating activity (○-----○) was assessed by adding 1-ml aliquots from every fourth tube into 40-ml cultures consisting of 60×10^6 (C58NT)D immune spleen lymphocytes and 2×10^6 mitomycin C-inactivated (C58NT)D lymphoma cells in EHAA medium. Cytotoxicity was determined after 5 days by using ^{51}Cr chromium-release assay. Relative lytic unit = lytic units per culture of cells cultured in the presence of the different column fractions − lytic units per culture of cells cultured in medium alone.

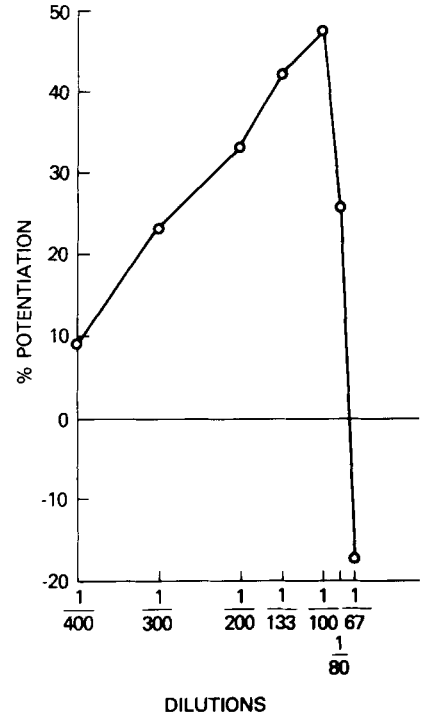

Figure 3. Dose-response effect of concentrated IPF on the generation of CTL. Percent potentiation $= \frac{a-b}{b} \times 100$. a = % cytotoxicity of CTL generated in the presence of IPF; b = % cytotoxicity of CTL generated in the absence of IPF.

obtained from the gel filtration column was divided into four parts and added to each of the immobilized enzymes. The mixtures were digested for 5 hr, and the supernatants obtained were dialyzed for 24 hr against PBS at 4°C. The data in Table

III clearly indicate that only the sample digested with DNAase lost its potentiating effect; digestion with RNAase or trypsin had no effect. These results suggest that the active molecule contains DNA. Positive controls, carried out by using calf thymus DNA, calf liver RNA, and glycogen phosphorylase b, indicated that all the immobilized enzymes were active. The digestion of DNA was determined by the increase in absorption at 260 nm and that of RNA by the decrease in optical density at 300 nm. Phosphorylase b digestion was followed by disc gel electrophoresis. Several double-blind experiments were performed in which the individual who performed the *in vitro* stimulation was given three media labeled only as a, b, and c. These tubes contained either media alone or media containing IPF or media containing DNAase-treated IPF. The results obtained in all cases confirmed the observations that the IPF contains DNA.

Agarose gel electrophoresis. The number of DNA species present in the purified sample was analyzed by slab gel electrophoresis in 0.7% agarose gel. Sample was applied to ten lanes and one lane was sliced off and stained with ethidium bromide. The rest of the slab was sliced across at areas corresponding to the three ethidium bromide-positive bands and homogenized in PBS. The supernatants obtained after centrifuging the gels were tested for the capability to potentiate the generation of cytotoxic effector cells. Figure 4 summarizes the results obtained. The low m.w. band was significantly the most active. Only a slight but significant amount of potentiation was observed at the middle band, and none could be demonstrated from the top of the gel. Similar results were obtained with another preparation. Digestion of the purified factor with DNAase before gel electrophoresis completely eliminated all the ethidium bromide reactive bands. This suggests that the bands observed were actually DNA and that IPF contains DNA. An ultraviolet spectrum analysis of the purified sample showed an absorbance ratio of 260 nm/280 nm of 1.6 with an optimum at 260 nm. Attempt to increase this value by subjecting the factor to 80% phenol extraction followed by three times ethanol precipitation of the aqueous phase was unsuccessful. The observation that the most active band is a small polynucleotide is consistent with the other data presented above, which suggest that IPF is a small m.w. material.

FIGURE IV

Potentiating Activity of IPF After Agarose Gel Electrophoresis

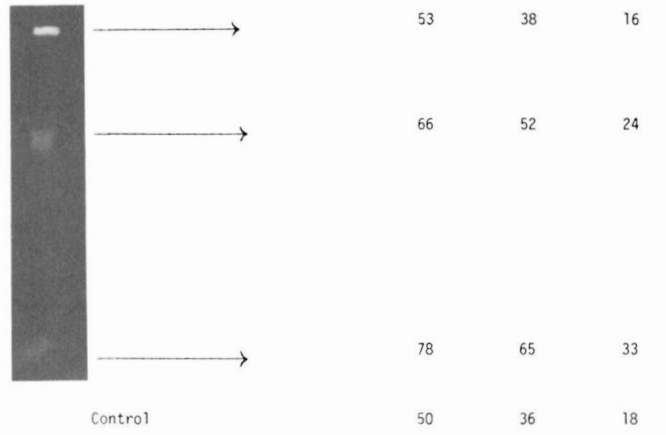

Figure 4. Potentiating effect of purified IPF after electrophoresis in 0.7% agarose gel. The arrows indicate areas that were sliced, homogenized, and assessed for potentiating activity. Statistical significance of the middle band material was $p < 0.02$ and for the lower band material, $p < 0.001$, as compared to control. Control culture consisted of (C58NT)D immune lymphocytes + (C58NT)D tumor cells cultured in medium alone.

TABLE II

Effect of inhibitors of protein and DNA syntheses on IPF elaboration

Source of Supernatant	Responding Cells	Stimulating Cells	% Cytotoxicity 30:1	10:1	1:1
A. BCG-primed cells + PPD	(C58NT)D spleen	(C58NT)D	96	90	54[a]
B. Mitomycin C (60 μg/ml) treated BCG-primed cells + PPD	(C58NT)D spleen	(C58NT)D	78	61	30
C. Puromycin (50 μg/ml) treated BCG-primed cells + PPD	(C58NT)D spleen	(C58NT)D	75	64	28
D.	(C58NT)D spleen	(C58NT)D	80	68	32
E.	(C58NT)D spleen	Syngeneic thymocytes	13	10	8

[a] Statistical significance of A as compared to B, C, or D at E/T of 10:1 was $p < 0.001$.

TABLE III

Effect of Enzymatic Degradation on Potentiating Factor

	Digestion	Cell-mediated Lympholysis (% Specific Cytotoxicity) 30:1	10:1	1:1
A. Culture medium alone		66	46	22
B. Culture medium + IPF		90	72	44
C. Culture medium + IPF	RNAase	88	70	42
D. Culture medium + IPF	Trypsin	89	70	38
E. Culture medium + IPF	DNAase	64	46	18

Statistical significance of B as compared to A at E/T of 30:1 was $p < 0.01$ and E as compared to B was $p < 0.001$.

DISCUSSION

The data reported here identify the nature of a soluble factor released by BCG-primed nonadherent lymphocytes which, when added to responding tumor-immune lymphocytes, augment the specific generation of cytotoxic effector cells against the sensitizing tumor. Similar potentiating effect was obtained when added to one-way mixed lymphocyte cultures of W/Fu lymphocytes responding to BN alloantigen (2). The IPF was released by BCG-immune lymphocytes only when restimulated by PPD. The data presented clearly suggest that IPF contains DNA. The discovery that the active factor contained DNA initially caused some concern, especially since most of the lymphokines that have been reported are protease-sensitive. In the present study, considerable amounts of time was expended searching for a protein factor until a spectrum of the purified factor was determined and found to show a 260-nm maximum absorption. Several experiments were performed in double-blinded fashion to test a) for the presence of a potentiating factor and b) whether the factor was DNAase-sensitive. These experiments conclusively confirmed the observations presented

above. Cell death or cell disintegration could not account for the possible source of this DNA, since the viability of the PPD-responding cells after 24 hr incubation was not significantly different from nonstimulated cultures. The observation that after treatment with puromycin or mitomycin C no potentiating activity could be found suggests that IPF production is dependent on intact metabolic activity and cellular division of the clone of lymphoid cells responding to PPD.

The exact size of the active DNA was not rigorously determined, but the data from gel filtration, agarose gel electrophoresis, and behavior on PM ultrafilters suggest that the m.w. must be small. The fact that it is excluded on PM-30 but retained on PM-10 ultrafilter would seem to place its size between 10,000 and 30,000. It should, however, be realized that these filters were calibrated with globular molecules, and the size exclusion may be different for rod-shaped molecules. After agarose gel electrophoresis, the most active band was close to the gel front, suggesting further that IPF is a small polynucleotide. The slightly active band in the middle of the agarose gel corresponds to m.w. of 1.5×10^6. It is very likely that this species is an aggregated form of the more active, faster migrating band, since DNA molecule of this size would have been excluded during the gel filtration chromatography (Fig. 1).

Although, several soluble products of lymphocyte activation have been described, most have been shown to be protease-sensitive (6). The observation that DNA is released by specifically stimulated tuberculin-sensitive lymphoid cells is therefore intriguing. Recently, other reports have demonstrated that rabbit spleen (7) and human blood lymphocytes stimulated with antigens (7–9) release newly synthesized DNA. As in the present study, cell death was ruled out as possible source of these extracellular DNA (8). Although lymphocytes release DNA, they have also been shown to take up DNA from the medium (7), thus suggesting this DNA interchange as a means of intercellular communication.

The mechanism by which DNA would potentiate the generation of cytotoxic effector cells specific to the immunizing tumor cell or alloantigen is not clear. Whether the effect is manifested by binding to the cell surface and acting as a messenger, more like some hormones, or whether it is actively pinocytosed to be transcribed into a specific mRNA and then to protein is not presently known. These types of questions can now be addressed more directly by using purified IPF. Some studies have been reported that suggest that in certain humoral immune responses synthetic homoribopolymer complexes of polyadenylic acid and polyuridilic acid (PolyA:U) are able to enhance the activation of T lymphocytes by antigen (10, 11). In one instance (11) the effect of poly A:U was attributed to the secretion of T cell helper factors and does not necessarily require the presence of antigen. Although this phenomenon has been observed *in vitro* (10–12) and also *in vivo* (13), the mechanism of its action is yet to be fully elucidated. It is likely that IPF and poly A:U share a common mechanism of action. However, this is only a speculation that needs to be further studied.

From the data obtained, it is possible that part of the adjuvant activity of BCG immunization resides in a DNA molecule released by specifically stimulated population of lymphoid cells. The potentiation of immune response by this molecule to tumor-specific antigen and alloantigen may be similar to the *in vivo* observation that BCG is a nonspecific immunostimulant. The characterization of the subset of lymphocytes elaborating IPF and that responding to the factor cannot presently be determined in the rat. In the mouse, similar factors are elaborated by Ly-1^+ helper T cell subpopulation and amplify the differentiation of Ly-23^+ prekiller T cells (14, 15). Nevertheless, the DNA molecule described in the present report should make the study of the mechanism of adjuvant activities possible under well-defined *in vitro* conditions and may prove useful when attempting to potentiate the generation of cytotoxic cells for immunotherapeutic purposes.

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