Biological Properties of Human Leukocyte Interferon Components

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(Accepted 20 December 1976)

SUMMARY

Human leukocyte interferon, purified approximately 1000-fold by affinity chromatography on immobilized anti-interferon globulins and SDS-Sephadex filtration, was resolved into one major and one minor component by adsorption chromatography on hydroxylapatite and electrophoresis in polyacrylamide gels. These components were indistinguishable in their capacity to protect bovine, porcine and murine cells, and the antiviral activities of both were equally susceptible to reduction by β-mercaptoethanol. They were neutralized to the same degree by rabbit anti-leukocyte interferon but were not neutralized by rabbit anti-fibroblast interferon serum. Mice immunized with either component developed antibodies to both but failed to form antibodies against human fibroblast interferon. Our present evidence indicates that the two components possess at most only minor structural and antigenic dissimilarities.

INTRODUCTION

Human leukocyte interferon preparations have been shown to exhibit considerable heterogeneity by a variety of methods. Several components with different charge properties have been described (Fantes, 1970a, b; Stanček, Gressnerová & Paucker, 1970; Mogensen et al. 1974). At least two molecular populations with interferon activity have been identified by means of Sephadex filtration (Fantes, 1970b), ion exchange chromatography (Matsuo, Hayashi & Kishida, 1974), elution from immobilized antibodies (Anfinsen et al. 1974), polyacrylamide gel electrophoresis under dissociating conditions (Stewart & Desmyter, 1975) and non-dissociating conditions (Borecký, Fuchsberger & Hajnická, 1974), or adsorption chromatography on hydroxylapatite (Törmä & Paucker, 1976), as well as by use of certain hydrophobic ligands (Chen et al. 1976). So far, two antigenically distinct constituents have been demonstrated, one of which being identical, or nearly so, with interferon produced in human embryonic fibroblasts and the other, found in dominant proportion, conforming to leukocyte specificity (Berg et al. 1975; Havell et al. 1975a; Havell, Berman & Vilček, 1975b; Paucker et al. 1975).

It is not certain whether the components defined by these diverse methods are in fact identical. However, they have been reported to differ in their antiviral activity on heterologous cell cultures. In one instance, the electrophoretic profile of human leukocyte interferon in polyacrylamide gels was resolved into two constituents, both active in human embryo cells, but only one major activity peak was discerned in mouse cell cultures which

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are also protected to some degree by human interferon (Borecký et al. 1974). In another study, two human leukocyte interferon populations eluted from SDS-polyacrylamide gels differed considerably in the ratio of their antiviral activities measured in human and rabbit cells (Stewart & Desmyter, 1975). When electrophoresis was carried out under reducing conditions, the more rapidly migrating component no longer had antiviral activity in human cells, although activity in cat cells was retained (Desmyter & Stewart, 1976). These results suggested that interferon components with distinctive configurational features may also behave differently not only in cells from other species but even in the homologous host. This notion derives support from the observation that mouse interferon obtained by immune-specific stimulation, which is known to differ antigenically as well as in stability properties from virus-induced mouse interferon (Youngner & Salvin, 1973), was superior as an anti-tumour agent (Salvin et al. 1975).

However, in a recent study from this laboratory, we were unable to document marked differences between two leukocyte interferon components isolated after several thousand-fold purification from hydroxylapatite (Törmä & Paucker, 1976). Exposure to β-mercaptoethanol reduced the antiviral activity in human cells of both components to a comparable level. Moreover, treatment with SDS appeared to destroy the activity of both components (as well as of rabbit interferon) in cultured rabbit cells (unpublished observations). We decided, therefore, to probe further into any differences that might exist between the two interferon species separated on hydroxylapatite with respect to (a) antiviral activities expressed in other hosts, (b) susceptibility of these effects to reduction of disulphide bonds by β-mercaptoethanol and (c) antigenic properties. This report describes our findings and conclusions.

**METHODS**

Production of interferon. Human leukocyte interferon was prepared at the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, as previously described (Strander & Cantell, 1966; Cantell, 1970). The interferon was concentrated at acid pH by KSCN (Cantell & Pyhältö, 1973; Fantes, 1974) and subsequently lyophilized. The specific activity was in the range of $1 \times 10^3$ units per mg protein. Occasionally, for use as antigen in neutralization tests, the leukocyte interferon was dissolved in acid ethanol (Fantes, 1970a) and fractionated at different levels of pH (Cantell et al. 1974), raising its specific activity to approximately $3 \times 10^5$ units per mg protein. Interferon was also obtained in cultures of human embryonic fibroblasts (FS-4 cells) by stimulation with poly (rI), poly (rC), according to Havell & Vilček (1972).

Affinity chromatography. Crude concentrated leukocyte interferon was partially purified on anti-interferon globulins coupled to Sepharose 4B (Cuatrecasas & Anfinsen, 1971). The antiseraum was prepared by hyperimmunization of a sheep with partially purified human leukocyte interferon (Mogensen, Pyhältö & Cantell, 1975) and obtained through the courtesy of Dr Kari Cantell, Helsinki, Finland. Chromatography was essentially carried out as described earlier (Ogburn, Berg & Paucker, 1973; Berg et al. 1975) except for the addition of 0·5 % crystalline bovine plasma albumin (BPA) to facilitate desorption from the antibody column. The specific activity of this preparation was in the order of $2 \times 10^5$ units per mg protein, mostly contributed by the added albumin.

Separation of interferon components. The techniques used for isolation of human leukocyte interferon components have been described elsewhere (Törmä & Paucker, 1976). In brief, interferon eluted from antibody columns was first treated with SDS at room temperature and then subjected to gel filtration on Sephadex G-100, also in the presence of SDS.
Interferon activity was eluted as a single homogeneous peak with quantitative recovery and an additional 20 to 50-fold purification. The eluted interferon was then chromatographed on hydroxylapatite according to Moss & Rosenblum (1972) with minor modifications cited previously (Törnä & Paucker, 1976). Interferon-containing fractions were concentrated by Aquacide (CalBiochem, La Jolla, California) and dialysed against 0.1% SDS in 0.01 M-sodium phosphate at pH 6.4. The interferon was applied to the column which had been equilibrated with the same buffer. The column was then washed with 0.1 M-sodium phosphate and developed with a linear gradient ranging from 0.1 to 0.5 M-sodium phosphate. All buffers contained 0.1% SDS and the pH remained constant throughout. The flow rate was 5 ml/hour, and fractions of 3.2 ml were collected. The entire procedure was carried out at room temperature. Computation of phosphate molarities was based on elution volumes and the assumption that the gradient was linear.

In some instances, individual peaks of interferon activity eluted from hydroxylapatite were also subjected to electrophoresis in SDS-polyacrylamide gels. The method adopted was modified from Weber & Osborn (1969). To preserve full interferon activity (Stewart & Desmyter, 1975), β-mercaptoethanol was omitted from the gels. Polymerized gels were pre-run to remove residual ammonium persulphate known to be injurious to interferon action (Fantes & Furminger, 1967). Samples and markers (BPA, chymotrypsinogen A, cytochrome c) were extensively dialysed against 0.01 M-sodium phosphate, pH 7, 0.1% SDS and loaded in 0.2 ml amounts. Bromophenol blue was used as tracking dye and electrophoresis was carried out at room temperature at a constant current of 8 mA/gel.

For measurement of interferon activity, 2 mm slices were extracted at room temperature for 48 h into 1 ml volumes of 0.01 M-sodium/potassium phosphate-buffered saline (PBS) with 0.1% SDS and 0.02% NaN3. Samples of 10 μl were diluted in PBS containing 0.5% BPA for interferon assay.

Interferon assays. Titrations in human (FS-4) cells were carried out in microplates according to Havell & Viléček (1972) with minor modifications specified elsewhere (Paucker et al. 1975). The highest dilution which protected half of the cells against vesicular stomatitis virus (VSV) was taken as one unit. Interferon titres are expressed in terms of the reference standard B69/19 for human interferon (National Institute for Biological Standards and Controls, London, England).

Protection of heterologous bovine turbinate (EBTr) cells (McClurkin et al. 1974), swine embryo testis (ST) cells (obtained from Dr A. McClurkin, National Animal Disease Center, Ames, Iowa) and mouse L-929 fibroblasts (Sanford, Earle & Likely, 1948) was measured in test tube cultures. Cells were exposed to serial twofold dilutions of human leukocyte interferon or interferon components and challenged with VSV 24 h later. End points were determined by partial inhibition of the c.p.e. of the challenge virus, as in microplates.

Interferon-neutralization assay. This test was performed in microplates as described above except that 50 μl volumes of serial fourfold dilutions of antisera were preincubated in the plates for 1 h at room temperature with 50 μl of graded interferon dilutions containing from one to 32 units, before addition of FS-4 cell suspension (Paucker et al. 1975). The highest dilution which neutralized 8 reference units of interferon by partially restoring virus c.p.e., corrected for 1 ml volume, represented the titre of the serum.

Immunizations. Antisera against crude human leukocyte and fibroblast interferons were produced in New Zealand white rabbits according to a schedule outlined earlier (Berg et al. 1975). In order to raise antibodies against individual human leukocyte interferon components A and B isolated from hydroxylapatite, random bred Swiss mice received a
RESULTS

Fractionation of human leukocyte interferon into two components

Essentially, two types of preparations were used in these studies. The first consisted of a human leukocyte interferon which had been pre-purified approximately 960 times from
**Human leukocyte interferon components**

Fig. 2. SDS-polyacrylamide gel electrophoresis of partially purified (see text) human leukocyte interferon (specific activity $5 \times 10^6$ units/mg protein). Overall gel length was 12 cm, electrophoresis time 9 h at 8 mA/gel and at room temperature. Panel I: profile obtained with interferon eluted from Sephadex after affinity chromatography. Panels II, III: profiles for pools A and B separated from this interferon on hydroxylapatite, as shown in Fig. 1. Arrows and numbers indicate fractions used as antigens in neutralization tests of Table 2 and for stability experiments listed in Table 4. Interferon, ○—○; molecular weight, ×——×.

acid ethanol (Cantell et al. 1974) and by two successive SDS-Sephadex filtrations as outlined under Methods. The interferon was then fractionated by adsorption chromatography on hydroxylapatite as shown in Fig. 1. Interferon activity was resolved into two distinct components termed A and B which eluted in the phosphate gradient at estimated molarities of 0.21 and 0.33. Extinction data indicated that some non-interferon proteins were segregated during this process, resulting possibly in a modest degree of additional purification. Total biological activity recovered in this instance was approximately 60 %, a minor portion of which (10 % of the original input) was contributed by peak A, and the remainder by peak B. Interferon-containing fractions in each of the two regions were pooled as indicated by the arrows, dialysed against 0.1 % SDS in PBS, distributed in small quantities and frozen at $-20^\circ$C. They were used to determine antiviral titres in several heterologous cell cultures (Table 1) and for immunization of mice (Table 3).

The second preparation originated from a crude leukocyte interferon which was pre-purified approximately 5000 times by two cycles of affinity chromatography on anti-interferon globulin coupled to Sepharose 4B, followed in turn by gel filtration on Sephadex G-100 and chromatography on SDS-hydroxylapatite. The isolated peaks A and B, as well as the initial Sephadex filtrate containing a mixture of the two components, were then subjected to electrophoresis in SDS-polyacrylamide gels as shown in Fig. 2. Panel I illustrates the distribution of interferon activities in the material before the hydroxylapatite step: two components were discerned with mobilities which corresponded to apparent molecular weights of 21000 and 16000, respectively. Separate electrophoresis of components A and B (panels II and III) confirmed that the major activity peaks migrated at different mobilities.
Table 1. Antiviral activities of human leukocyte interferon components in cells from other species

<table>
<thead>
<tr>
<th>Human interferon preparation</th>
<th>L-929</th>
<th>ST</th>
<th>EBTr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^5 units*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>470</td>
<td>213</td>
<td>5800</td>
</tr>
<tr>
<td>Native, SDS-treated</td>
<td>130</td>
<td>769</td>
<td>8000</td>
</tr>
<tr>
<td>Hydroxylapatite peak A</td>
<td>470</td>
<td>213</td>
<td>4000</td>
</tr>
<tr>
<td>Peak B</td>
<td>300</td>
<td>333</td>
<td>2000</td>
</tr>
</tbody>
</table>

* Interferon activity not affected by SDS treatment or hydroxylapatite chromatography.
† Mouse fibroblasts (L-929), swine embryo testis (ST) and bovine turbinate (EBTr) cells.
‡ Protective activity in human (FS-4) cells: protective activity in heterologous cells.

corresponding fairly closely to those detected in panel I. The extracted gel fractions were kept frozen at -20 °C and those identified by number were used as antigens in neutralization tests (Table 2) or to assess the effect of reduction of disulphide bonds on antiviral protection in non-human cell cultures (Table 4).

Heterospecific activities of human leukocyte interferon components

In a first series of experiments, the effects of leukocyte interferon components A and B eluted from hydroxylapatite were compared in three lines of mammalian cells which had previously been identified as sensitive to human interferon (Levy-Koenig, Golgher & Paucker, 1970; Borecký et al. 1974; Gresser et al. 1974). Interferon titres were determined in EBTr, ST and L-929 cell cultures and related to the antiviral effects recorded in human cells. For easier comparison, all values were corrected on the basis of 1 x 10^5 units expressed in the human system. Both native and SDS-treated crude leukocyte interferon preparations were included as controls, to detect any changes that may have occurred during treatment with the detergent and purification sequence. The results are shown in Table 1. They indicate that EBTr cells were the most sensitive and L cells the least sensitive to the human interferon materials tested, with ST cells occupying an intermediate position. Hydroxylapatite peaks A and B possessed comparable activities in the three systems under study, well within reliability limits of the interferon assay. Neither of the purification or separation steps nor treatment with SDS significantly altered the protective ratios established for each cell system.

Antigenic specificities of human leukocyte interferon components

The antigenic properties of leukocyte interferon components A and B were next compared by means of monospecific anti-human interferon sera prepared in rabbits injected with either leukocyte or FS-4 interferon. Results of neutralization tests with the gel eluates identified in Fig. 2 and corresponding controls are presented in Table 2. The data demonstrate that the leukocyte interferon antigens, either before or after hydroxylapatite chromatography, and regardless of their electrophoretic mobility in SDS-polyacrylamide gels, were neutralized only by rabbit anti-leukocyte interferon serum but not by anti-fibroblast interferon serum. No cross-neutralization between leukocyte and fibroblast interferon antigens was observed. Therefore, neither of the leukocyte interferon components characterized on SDS-polyacrylamide gels was identical with the fibroblast-specific factor previously shown to reside...
Human leukocyte interferon components

Table 2. Antigenic specificity of human leukocyte interferon components eluted from SDS-polyacrylamide gels

<table>
<thead>
<tr>
<th>Interferon antigen</th>
<th>Gel fraction*</th>
<th>Neutralizing titre/ml with antisera against</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-Sephadex filtrate</td>
<td>36</td>
<td>Le IF† 3200 &lt; 100</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>FS-4 IF‡ &lt; 100</td>
</tr>
<tr>
<td>Hydroxylapatite peak A</td>
<td>39</td>
<td>Le IF† 3200 &lt; 100</td>
</tr>
<tr>
<td>peak B</td>
<td>36</td>
<td>FS-4 IF‡ 1600 &lt; 100</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>ethanol-purified Le IF† 3200 &lt; 100</td>
</tr>
<tr>
<td>Non-purified FS-4 IF</td>
<td>—</td>
<td>&lt; 100 1200</td>
</tr>
</tbody>
</table>

* See Fig. 2.
† Human leukocyte interferon.
‡ Human fibroblast interferon.

Table 3. Antigenicity of human leukocyte interferon components

<table>
<thead>
<tr>
<th>Mouse no.*</th>
<th>Immunizing interferon†</th>
<th>Neutralizing antibody titre/ml against 8 units of test interferon antigens‡</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Peak A</td>
<td>Le 2560 A 1280 B 640</td>
</tr>
<tr>
<td>2</td>
<td>Peak A</td>
<td>Le 2560 A 1040 B 640</td>
</tr>
<tr>
<td>3</td>
<td>Peak B</td>
<td>Le 640 A 640 B 320</td>
</tr>
</tbody>
</table>

* Mouse no. 4 immunized with peak B failed to respond.
† Obtained by hydroxylapatite chromatography (see Fig. 1).
‡ Corresponding to non-purified leukocyte interferon (Le) and hydroxylapatite peaks A and B.

in human leukocyte interferon preparations (Berg et al. 1975; Havell et al. 1975a, b; Paucker et al. 1975).

However, the possibility remained that the rabbit anti-leukocyte interferon serum harboured mixed antibody populations with neutralizing activities directed against either one or the other component. In that event, antigenic dissimilarities of the two components might have gone undetected. We decided, therefore, to immunize mice against each of the components isolated on hydroxylapatite. Paucity of the material available permitted the inoculation of only four animals, two with peak A and two with peak B. Neutralizing antibodies developed after 5 weeks in three of the animals, and terminal bleedings were obtained 3 weeks later, i.e. after the mice had received a total of eight weekly injections. Sera were assayed for neutralization of each of the immunizing antigens as well as of a non-purified leukocyte interferon control antigen. According to the data shown in Table 3, one mouse in each group had formed antibodies which neutralized all three antigens equally well, indicating that peaks A and B were antigenically quite similar. However, one animal (No. 2) neutralized the immunizing antigen (peak A) at a considerably higher titre than peak B. This observation would indicate that the two components may also possess some distinct antigenic determinants which can be recognized only by immune sera from particularly responsive mice. The titre against the leukocyte interferon control antigen was in the intermediate range. This would seem to suggest that the control interferon preparation consisted predominantly of component B against which only low levels of antibody were produced in this particular animal.
Table 4. Effect of \(\beta\)-mercaptoethanol on protective activities of human leukocyte interferon components in human and other mammalian cells

| Interferon preparation | Treatment† | Protective activity* FS-4 (Units) (Units (%) | L-929 (Units) (Units (%) | ST (Units) (Units (%) | EBTr (Units) (Units (%)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Native SDS/U</td>
<td>100000</td>
<td>100 (100)</td>
<td>200 (100)</td>
<td>80000 (100)</td>
<td>4000000 (100)</td>
</tr>
<tr>
<td>SDS/ME/U</td>
<td>8000</td>
<td>(8)</td>
<td>200 (100)</td>
<td>1280 (16)</td>
<td>120000 (3)</td>
</tr>
<tr>
<td>Peak A (1)‡</td>
<td>SDS</td>
<td>8000 (100)</td>
<td>60 (100)</td>
<td>&gt;640</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SDS/ME/U</td>
<td>1600 (20)</td>
<td>80 (&gt;100)</td>
<td>160 (&lt;25)</td>
<td>—</td>
</tr>
<tr>
<td>(2)‡</td>
<td>SDS</td>
<td>3000 (100)</td>
<td>—</td>
<td>—</td>
<td>80000 (100)</td>
</tr>
<tr>
<td></td>
<td>SDS/ME/U</td>
<td>&lt;200 (&lt;7)</td>
<td>—</td>
<td>—</td>
<td>2000 (3)</td>
</tr>
<tr>
<td>Peak B (3)‡</td>
<td>SDS</td>
<td>32000 (100)</td>
<td>60 (100)</td>
<td>&gt;640</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SDS/ME/U</td>
<td>6400 (20)</td>
<td>40 (67)</td>
<td>160 (&lt;25)</td>
<td>—</td>
</tr>
<tr>
<td>(4)‡</td>
<td>SDS</td>
<td>20000 (100)</td>
<td>—</td>
<td>—</td>
<td>1000000 (100)</td>
</tr>
<tr>
<td></td>
<td>SDS/ME/U</td>
<td>200 (1)</td>
<td>—</td>
<td>—</td>
<td>2000 (2)</td>
</tr>
</tbody>
</table>

* Measured in human fibroblasts (FS-4), mouse fibroblasts (L-929), swine embryo testis (ST) and bovine turbinate (EBTr) cells.
† Abbreviations denote Na dodecyl-SO₄ (SDS), \(\beta\)-mercaptoethanol (ME), urea (U). Treatment was in 1% SDS, 5 M-urea in the presence or absence of 1% ME, for 1 min at 100°C, followed by dialysis against Na/K phosphate-buffered saline containing 0.1% SDS.
‡ For identification of fractions see Fig. 2. (1) = fraction 39-40, panel II; (2) = fraction 36, panel I; (3) = fraction 41, panel III; (4) = fraction 41, panel I.

**Effect of reduction on protective activities expressed in heterologous cells**

According to a previous report, reduction by \(\beta\)-mercaptoethanol may affect the behaviour of interferon molecular species in homologous and heterologous cell systems in different ways (Desmyter & Stewart, 1976). Therefore, we examined the antiviral activities of the two interferon components eluted from SDS-polyacrylamide gels in various cell cultures, both before and after exposure to the reducing agent. These data are recorded in Table 4. The results show that treatment with \(\beta\)-mercaptoethanol partially destroyed the antiviral expression of both components as well as of a leukocyte interferon control preparation in human, swine and bovine cell cultures. However, the degree of inactivation was to some extent variable. On the other hand, antiviral activity for mouse cells was fully preserved under the same conditions in the case of all three interferon preparations. Therefore, within the limitations of these experiments, cross-protection of cells from heterologous hosts by both leukocyte interferon components appeared to be comparably influenced by treatment with the reducer.

**DISCUSSION**

The present study was undertaken to clarify the differences in antiviral expression which have been associated with two distinct molecular species of human leukocyte interferon. We found that the protective behaviour of both components in relation to the titre in human cells was not significantly different when examined in three heterologous cell systems. The magnitude of the antiviral effects noted corresponded in general quite well to that recorded with mixed populations of leukocyte interferon components in bovine and porcine cells (Gresser et al. 1974) as well as in mouse cells (Levy-Koenig et al. 1970; Borecký et al. 1974).

Human leukocyte interferon preparations are known to harbour activity that is anti-
genically related to human fibroblast interferon (Berg et al. 1975; Havell et al. 1975a, b; Paucker et al. 1975). However, neutralization tests with monospecific rabbit anti-interferon sera disclosed that neither component possessed fibroblast specificity. The small number of mice that could be injected with each component precluded a more precise antigenic analysis. Within these limitations, however, it is apparent that at best only minor antigenic differences exist, and these might be recognized only with sera from hyperresponsive animals. This is also the first demonstration that antibodies against an interferon can be raised in that species. The advantages of mice for the study of serological relationships among interferons will be discussed elsewhere.

Examination of reduced preparations of extensively purified leukocyte interferon components in the different cells under study failed to uncover any marked differences in their antiviral behaviour. Reduction by β-mercaptoethanol partially destroyed the protective activity measured in human, bovine and porcine cells, whereas the low degree of protection evident in mouse cells was fully preserved. The degree of inactivation appeared to differ more with individual preparations or experimental technique rather than with a given molecular interferon species. The data also suggest that if protection of heterologous cells indeed turns out to be an expression of single molecules (Paucker et al. 1975), the sites responsible for such interaction may reside in the same basic structure and be subject to simultaneous inactivation when disulphide bonds are ruptured.

The data presented here lead to the conclusion that two molecular populations found in approximately 1000-fold purified human leukocyte interferon possess no major differences in their antigenic make-up and in their protective behaviour in those heterologous cell cultures that we examined. Moreover, when the growth inhibitory effects of companion gel eluates of peaks A and B (corresponding to panels II and III of Fig. 2) were examined in human cells, they were found to be comparable in relation to their antiviral activity (H. Strander, personal communication). This does not, of course, preclude differences observed in other types of cells, for example as described by Stewart & Desmyter (1975).

As to their origin, the two interferon populations may originate from different cell types in the leukocyte suspension (Epstein, Kreth & Herzenberg, 1974). Alternatively, they may have arisen during prolonged storage at low pH as suggested by recent observations of others (Berg & Fantes, personal communication). Studies on the possible source of the different components in leukocyte interferons are now in progress.

This investigation was supported in part by Contract NOI-AI-52520 from the U.S. Public Health Service. The authors thank the Finnish Red Cross Blood Transfusion Service for its generous contribution to this programme.

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(Received 19 November 1976)