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Production and characterization of bovine interleukin-2

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Summary. A lymphokine analogous to interleukin-2 from other species was generated from bovine suprapharyngeal lymph nodes and characterized biochemically. The isolated material can support the long-term growth of concanavalin A (Con-A) or mixed lymphocyte reaction (MLR)-activated bovine T cells. The material elutes from DEAE-Sephadex at a low salt concentration, approximately 0·075 M, and exhibits a molecular weight of approximately 25,000 on Sephadex G-100. When subjected to analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), three peaks of activity were observed, corresponding to molecular weights of 14,400, 16,800 and 20,200. Finally, the isolated material exhibited a marked heterogeneity when subjected to chromatofocussing. Three major peaks of activity, with pIs of approximately 5·95, 5·41 and 5·0 are present with peaks of lesser activity at pIs of 5·82, 5·70, 4·73 and 4·20.

INTRODUCTION

Interleukin-2 is a soluble lymphokine which functions to mediate the proliferative response of activated T cells (Watson *et al.*, 1979; Gillis, Smith & Watson, 1980). Interleukin-2 is an antigen-non-specific and genetically non-restricted lymphokine which is produced by T cells following stimulation with antigen, mitogen or alloantigen (Watson & Mochizuki, 1980; Smith, 1980). It has been shown to participate in a number of *in vitro* immune responses including the maintenance of functional T-helper and cytotoxic long-term cell lines, enhancement of thymocyte mitogenesis, and the induction of cytotoxic T lymphocytes in thymocyte and nude spleen cell populations (Watson *et al.*, 1979; Gillis *et al.*, 1980). Interleukin-2 (IL-2) from human and murine systems has now been purified to apparent homogeneity (Gillis *et al.*, 1982). Despite the major economic importance of cattle as a food animal, the bovine immune system is only beginning to be investigated, and little is known about lymphokines in the bovine system. Unfortunately, bovine lymphocytes are not reactive with IL-2 preparations from a number of other species and proliferate only in the presence of bovine-derived factors. This necessitates the production and characterization of bovine IL-2 as a prerequisite to the establishment of long-term IL-2-requiring bovine T cell lines. This work represents our initial attempts at the isolation and characterization of bovine IL-2.

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MATERIALS AND METHODS

IL-2 production

The medium used throughout (M-DMEM) was prepared as described by Iscove & Melchers (1978), with the exception that no soybean lipid was added. In short, Dulbecco's modified Eagles medium was supplemented with: L-alanine (25 mg/litre), L-asparagine

(25 mg/litre), L-aspartic acid (30 mg/litre), L-cystine (22 mg/litre), L-glutamic acid (75 mg/litre), L-proline (40 mg/litre), sodium pyruvate (220 mg/litre), vitamin B_{12} (0·013 mg/litre), biotin (0·013 mg/litre), sodium selenite (0·0173 mg/litre) and 2-mercaptoethanol at 5×10^{-5} M. Additionally, delipidated bovine serum albumin was added at 0·5 mg/ml and human transferrin with Fe^{+++} ion supplemented to 0·33 of saturation added at 1 μg/ml.

Lymphocytes were obtained from the suprapharyngeal lymph nodes of freshly slaughtered cattle and prepared utilizing a modification of the method of Peters (1975). In brief, the lymph nodes were minced and pressed through a coarse stainless steel screen and washed with M-DMEM containing 10% calf serum (CS). The cells were suspended in M-DMEM–10% CS and applied to a glass wool column equilibrated with the same medium. The cells were run into the column and allowed to incubate at room temperature for 10 min and then eluted with the same medium. The cells were collected and washed several times in M-DMEM and resuspended to 4×10^6 cells/ml in the same medium. Fresh concanavalin A (Con A) prepared by the method of Agrawal & Goldstein (1967) was added to a concentration of 2 μg/ml and the conditioned medium (CM) harvested for IL-2 purification after 24–28 hr of incubation of the cells at 37° in a 5% CO_2 atmosphere.

IL-2 assay

The assay system used was an adaptation of the quantitative microassay of Gillis *et al.* (1978). Interleukin-2-responsive T cell blasts were generated utilizing either Con A stimulation (Larsson, Iscove & Coutinho, 1980; Anderson *et al.*, 1979) for an allogeneic mixed lymphocyte reaction. Lymph node lymphocytes at 4×10^6 cells/ml in M-DMEM supplemented with 2·5% calf serum were stimulated for 4 days with 2 μg/ml Con A. The blasts were counted and reseeded to a density of 1×10^5 blasts/ml in 50% CM/50% M-DMEM containing 2·5% calf serum and propagated by repeated seedings every 3–4 days. After approximately 10–14 days in culture the blasts were used as a source of IL-2-responsive cells in the microassay. Interleukin-2-requiring blasts were also prepared in an allogeneic mixed lymphocyte reaction by incubating 2×10^6/ml responding cells and 2×10^6/ml mitomycin C-treated stimulator cells for 6 days in M-DMEM containing 2·5% calf serum. The stimulator cells were suspended at 1×10^7/ml in M-DMEM and 100 μg/ml mitomycin C (Sigma) added. After incubation at 37° and 5% CO_2 for 45 min, the cells were washed three times in M-DMEM and added to the responding cells at 2×10^6/ml. The resulting blasts were propagated by seeding at 5×10^4 cells/ml every 3–4 days in M-DMEM containing 2·5% calf serum and 5 units/ml of Con A-free IL-2. The Con A-free IL-2 was obtained by partial purification of CM utilizing ammonium sulphate fractionation, ion exchange chromatography and gel filtration. After 2 weeks in culture the blasts were then used in the microassay.

In the assay proper, 20 μl of the sample to be assayed or serial dilutions of the sample were dispensed into individual wells of a microtitre plate and 180 μl of M-DMEM–2·5% calf serum containing 1×10^5 blasts was added to each well. After 20 hr of incubation each well was pulsed for 4 hr with 1 μCi of [^{3}H]thymidine. The cells were harvested on glass fibre strips and thymidine incorporation determined by scintillation counting. Units of activity are defined by assuming that a preparation containing 1 unit/ml will produce half the maximal proliferative response in the standard assay system.

DEAE-Sephadex ion-exchange chromatography

The bovine lymphocyte conditioned medium was first concentrated by ammonium sulphate fractionation. The fraction precipitating between 50 and 90% saturation was dialysed against 0·01 M Tris, pH 7·8 and applied to a 2·5 × 18 cm column of DEAE-Sephadex equilibrated in the same buffer. A linear salt gradient from 0·0 to 0·5 M NaCl was applied (total volume 400 ml) and 20 μl of each 7 ml fraction was assayed for proliferative activity in the standard microassay.

Sephadex G-100 gel filtration chromatography

The active fractions obtained from the DEAE-Sephadex column were pooled and concentrated to 10 ml using an Amicon UM-10 ultrafiltration membrane. The sample was applied to a 2·5 × 140 cm column of Sephadex G-100 equilibrated with phosphate-buffered saline (pH 7·3) containing 0·1% polyethylene glycol. Elution was carried out with the same buffer. Fractions containing proliferative activity were pooled and concentration with an UM-10 ultrafiltration membrane.

SDS-PAGE analysis

Analysis of the Sephadex G-100 purified material by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by

Gillis *et al.* (1982). A suitable aliquot of the partially purified IL-2 (100 units) was dialysed against 50 mM NH_4HCO_3 and lyophilized. The pellet was dissolved in a small volume of Laemmli sample buffer (1% SDS and 10% glycerol) and heated at 60° for 15 min. The sample was electrophoresed in a 12·5% Tris-glycinate discontinuous gel until the tracking dye had migrated off the gel. The IL-2-containing lane was removed and sliced into 1·5 mm slices and each slice placed in an individual well of a microtitre plate. The individual slices were washed twice with M-DMEM containing 2·5% calf serum, crushed with a small sterile rod and incubated overnight in 100 μl of M-DMEM containing 2·5% calf serum at 37°. The microtitre plate was then chilled at 4° for 4 hr and centrifuged at 400 *g* for 15 min. Aliquots of the individual supernatants were then assayed for proliferative activity in the standard microassay.

Chromatofocusing

Chromatofocusing was performed essentially as described by the manufacturer Pharmacia. A suitable aliquot of the Sephadex G-100 purified material (100 units) was dialysed against 0·025 M histidine buffer, pH 6·2, and applied to a 0·75 × 25 cm column of Polybuffer Exchanger 94 previously equilibrated with the same buffer. The pH gradient was developed by applying 100 ml of Polybuffer 74 previously titrated to pH 4·0. Samples (2 ml) were collected, the pH was measured, and the samples were dialysed overnight against saturated ammonium sulphate to remove the Polybuffer 74. The samples were then dialysed against phosphate-buffered saline (pH 7·3) containing 0·1% polyethylene glycol 6000 and assayed for proliferative activity in the standard microassay.

RESULTS

IL-2 assay

The determining factor for a dependable IL-2 assay was the assay cells themselves. Since we were not able to demonstrate any IL-2 cross-reactivity between the bovine system and the other more thoroughly studied species, it was necessary to generate bovine IL-2-requiring cells. Accordingly, T cell blasts were generated by Con A stimulation and by allogeneic mixed lymphocyte reactions and evaluated for their usefulness in the IL-2 microassay. Both Con A- and MLR-activated blasts gave similar and reproducible results in the microassay. Whereas the MLR-activated blasts propagated in Con A-free IL-2 preparations became unresponsive to Con A stimulation within 10–14 days, the Con A-activated blasts retained the ability to respond to Con A for as long as 6–7 weeks after initiation of culture. The MLR-derived blasts were required, therefore, for the assay of unfractionated Con A supernatants, while blasts derived by either technique were suitable for assay on Con A-free, fractionated IL-2 preparations.

A continually recurring problem, noted often by others, in the maintenance of T cell blasts has been a 'crisis period' occurring about 14 days after initiation of culture. The crisis period was observed regardless of how the lymphocytes had been activated and was characterized by a rapid decline in cell viability even in the presence of excess IL-2. It was observed, however, that the cells in crisis period would recover following an appropriate restimulation. Restimulation of the MLR blasts with an equal number of stimulator cells restored the ability of the blasts to repsond to unfractionated CM or partially purified IL-2. In fact, it was observed that maintenance of the MLR blasts over an extended period of time required repeated allogeneic stimulation approximately every 14 days. Cells activated by Con A stimulation would recover from the crisis following restimulation with Con A at a level of 0·1 μg Con A/1 × 10^5 blasts/ml. The blasts regained their ability to respond to IL-2 preparations and could be propagated for extended periods in IL-2-containing CM. Occasionally the blasts also required a second restimulation with Con A before extended periods of growth could be achieved. We have been able to maintain IL-2-responsive blasts in this way for as long as 8 months.

DEAE-Sephadex ion-exchange chromatography

The CM was first fractionated and concentrated by precipitation with ammonium sulphate. Sequential precipitations showed that essentially all of the proliferative activity was recovered between 60 and 80% saturation (unpublished observation). Routinely, the CM was subjected to a 50–90% treatment, a procedure which removes greater than 95% of exogenously added ^{125}I-Con A (unpublished observations). After equilibration by dialysis, the material was applied to a DEAE-Sephadex column and developed with a linear salt gradient as shown in Fig. 1. The proliferative activity eluted at a low salt concentration with a single peak of activity centered at approximately 0·075 M NaCl. The peak was rather broad, however, and

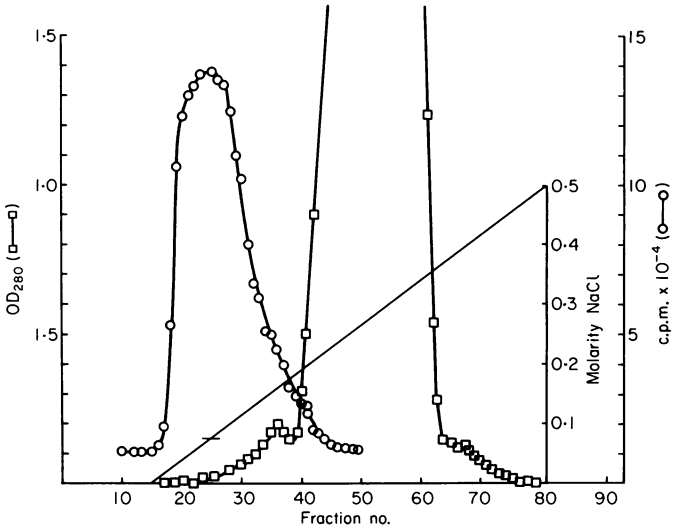

Figure 1. DEAE-Sephadex ion-exchange chromatography. The 50–90% ammonium sulphate fraction from 1 litre of conditioned media was dialysed against 0·01 M Tris, pH 7·8, and applied to a 2·5 × 18 cm column of DEAE-Sephadex equilibrated in the same buffer. A linear salt gradient from 0·0 to 0·5 M NaCl was applied to the column and 20 μl of each 6 ml fraction was assayed for proliferative activity. The results are expressed as the c.p.m. obtained in the standard microassay.

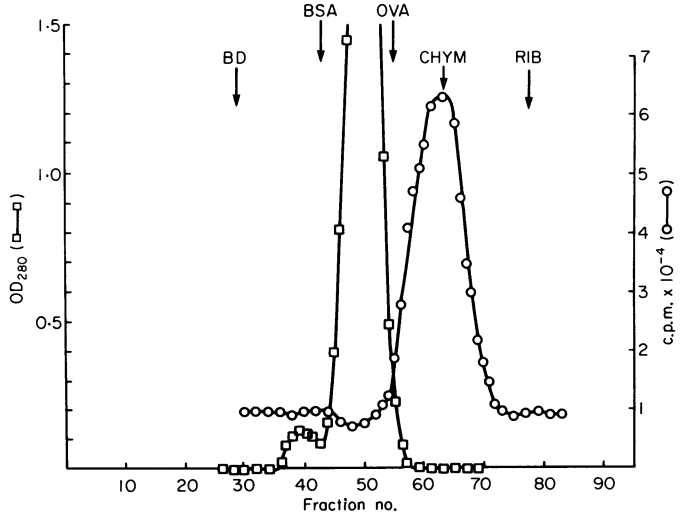

Figure 2. Sephadex G-100 gel filtration chromatography. The active fractions following ion-exchange chromatography were pooled, concentrated and applied to a 2·5 × 140 cm column of Sephadex G-100 equilibrated in PBS containing 0·1% polyethylene glycol. After elution with the same buffer each 8 ml fraction was sterilized by filtration and assayed for proliferative activity. The results are expressed as the c.p.m. obtained in the standard microassay.

encompassed a range of NaCl concentrations of from approximately 0·05 M to 0·2 M.

Sephadex G-100 gel filtration chromatography

The active fractions from the DEAE-Sephadex column were concentrated to 10 ml and applied to a Sephadex G-100 column (Fig. 2). The activity eluted as a single broad peak with an apparent average molecular weight of 25,000. Marterial purified by Sephadex G-100 was used a source of IL-2 for the maintenance of assay cells and also as a source of material for further characterization. The levels of activity and the yields obtained through the Sephadex G-100 step are shown in Table 1. Greater than 70% recoveries are obtained, and the material is stable for many months (unpublished observation) at this stage.

Table 1. IL-2 recovery and yield

	Units/liter	% Recovery
Conditioned medium	12,800	100
Ammonium sulphate	12,000	93
DEAE-Sephadex	11,247	87
Sephadex G-100	9,200	71

Conditioned media was generated by incubating 4×10^6 lymphocytes/ml with 2 μg/ml Con A for 24 hr. The conditioned media was fractionated as detailed in the text. An aliquot from each stage of the fractionation was frozen and proliferative activity of all samples were determined simultaneously. The results are quantitated by probit analysis as described by Gillis *et al.* (1978).

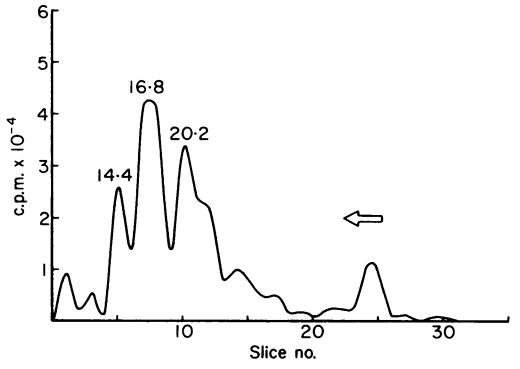

Figure 3. Analysis of IL-2 by SDS-PAGE. A 100 μl aliquot of the G-100 purified IL-2 was dialysed against 50 mM NH_4NCO_3 and lyophilized. The lyophilized pellet was dissolved in a small volume of Laemmli sample buffer containing 1% SDS and 10% glycerol and heated at 60° for 15 min. The sample was electrophoresed on a 12·5% Tris-glycinate discontinuous gel as described by Laemmli & Favre (1973). The IL-2-containing lane was excised, sliced, and the IL-2 eluted from each slice into M-DMEM containing 2·5% calf serum. A 20 μl aliquot of each sample was assayed in the microassay and the results expressed as the c.p.m. obtained in the standard microassay.

SDS-PAGE

The G-100-purified material was subjected to assay by SDS-PAGE (Fig. 3). Electrophoresis on 12·5% acrylamide gels demonstrated a marked, but repeatable heterogeneity. Three major peaks of activity were obtained corresponding to molecular weights of approximately 14,400, 16,000 and 20,200 as determined by comparison with molecular weight standards run in parallel. This type of heterogeneity has been observed in other species (Robb & Smith, 1981) and is very likely due to variations in the glycosylation of the molecule. The recoveries of biological activity are good and usually fall in the 40–50% range (data not shown).

Chromatofocusing

The G-100-purified material was subjected to chromatofocusing rather than isoelectric focusing because of the convenience and speed of the technique and the higher recoveries obtained. The profile of proliferative activity is shown in Fig. 4. Three major peaks of activity were found corresponding to pH values of 5·95, 5·41 and 5·0. Lesser peaks of activity appeared at pH values of 5·82, 5·70, 4·73 and 4·2. As indicated by the manufacturer, the apparent pI vaues are slightly low, and in our experiments bovine serum albumin eluted at 0·1 to 0·2 pH units lower than literature values. The values obtained for IL-2 can still be used as an approximation of the actual pI values. No biolgical activity was detected above a pH of 6 and pH gradients were routinely used between pH 6 and 4. Although handling losses are magnified because of the number of peaks, we could still recover from 30 to 40% of the applied material.

DISCUSSION

This study examined the production and initial characterization of a lymphokine from bovine lymph node

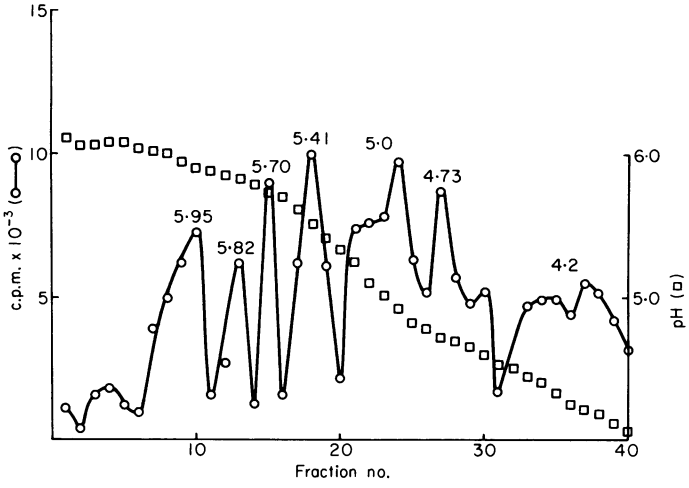

Figure 4. Analysis of IL-2 by chromatofocussing. A 100 unit aliquot of Sephadex G-100-purified IL-2 was equilibrated in 0·025 M His-HCl, pH 6·2, and applied to an 0·75 × 25 cm column of Polybuffer Exchanger 94 (Pharmacia Fine Chemicals) pre-equilibrated in the same buffer. A pH gradient was established by applying 100 ml of Polybuffer 74 (pH 4·0) at a flow rate of 10 ml/hr. After measuring the pH of each 2 ml sample, the polybuffer was removed by dialysis and the proliferative activity in each sample determined in the standard microassay. The results are expressed as the c.p.m. obtained in the standard microassay.

lymphocytes. The cow is an extremely important food animal and studies aimed at the immunological resistance of cattle to disease could be of great economic importance. Although it has become apparent in the last few years that soluble mediators of immunoregulation such as lymphokines and monokines can have potent regulatory effects in the various immune responses, very little information has appeared on lymphokines in the bovine system. Recently, Baker & Knoblock (1982a, b) reported their results in the production and Sephadex G-100 purification of a bovine costimulator from bovine peripheral blood lymphocytes. This factor appears to be the bovine equivalent of interleukin-2 and has the ability to promote the extended culture of T cell mitogen-activated lymphocytes. However, only limited numbers of lymphocytes are conveniently available from bovine peripheral blood.

Utilizing bovine suprapharyngeal lymph nodes large quantities of lymphocytes ($1·2 \times 10^{10}$ cells/pair lymph nodes) are available to generate sufficient quantities of conditioned medium to allow the biochemical characterization of the resultant lymphokines. We have extended the observations of Baker & Knoblock (1982a, b) and characterized a lymphokine analogous to interleukin-2 by ammonium sulphate fractionation, anion exchange chromatography, gel filtration chromatography, SDS-PAGE, and chromatofocusing. Bovine IL-2 precipitates in the same general range of ammonium sulphate concentrations, between 60 and 80% saturation, as detailed in other species (Gillis *et al.*, 1980). It elutes from DEAE-Sephadex with a single peak centered at approximately 0·075 M NaCl and ranging from approximately 0·05 to 0·2 M NaCl, while mouse IL-2 elutes at approximately 0·185 M NaCl and rat and human IL-2 does not bind to DEAE-cellulose in 0·05 M sodium phosphate buffer (pH 7·6) (Gillis *et al.*, 1980). On the basis of ionic properties, the bovine IL-2 would seem to more closely resemble mouse IL-2. The bovine IL-2 routinely exhibited a molecular weight of approximately 25,000 on Sephadex G-100, while mouse IL-2 exhibited a molecular weight of 30,000–32,000, and human and rat 15,000–17,000. Robb & Smith (1981) has demonstrated that human IL-2 derived from the JURKAT cell line resolves into two peaks of activity on SDS-PAGE with nominal molecular sizes of 16,200 and 14,200.

When bovine IL-2 was subjected to SDS-PAGE we were able to recover peaks of activity with apparent molecular sizes very close to those found by Robb & Smith (1981), namely 16,800 and 14,400. In addition,

we observed a third peak of activity with an apparent molecular size of 20,200. Very likely these multiple peaks of activity are due to variations in the glycosylation state of the IL-2 molecule.

Human IL-2 has been shown to have a pI value between 6·0 and 6·5, rat IL-2 a pI between 5·4 and 5·6, and mouse IL-2 a pL between 4·3 and 4·9 (Gillis *et al.*, 1980). Analysis of bovine IL-2 by chromatofocusing resulted in a surprisingly large number of peaks of activity with pIs ranging from approximately 6 to 4. The major peaks of activity exhibited pIs of approximately 5·95, 5·41 and 5·0. When larger amounts of material (100 units of activity) were applied to the column, additional lesser peaks of activity appeared at pIs of approximately 5·82, 5·70, 4·73 and 4·2. This activity profile exhibits more heterogeneity than the isolectric focusing profiles established for human, rat and mouse (Gillis *et al.*, 1980). This increased heterogeneity appears to be a function of the increased resolving power of the chromatofocusing technique, as mouse IL-2 purified from superantants of the EL-4 cell line exhibited as many as nine peaks of activity ranging from pIs of 4·6 to 3·4 when analysed by chromatofocusing (Pure *et al.*, 1982). Robb & Smith (1981) have resolved human IL-2 into three peaks of activity by isoelectric focusing, and shown that the heterogeneity was due to variations of sialic acid content. Whether this level of heterogeneity can be explained by variations in sialic acid content remains to be determined. If this was the case, the IL-2 would have to contain unusually high amounts of carbohydrate and/or sialic acid. Recently Gootenberg, Ruscetti & Gallo (1982) have established several cutaneous T cell lymphoma-leukaemias which constitutively produce IL-2. This tumour cell-derived IL-2 has quite different biochemical properties than IL-2 derived from normal lymphocytes, although both are biologically active. The tumour-derived IL-2 has a more acidic pI than IL-2 from normal lymphocytes, and the shift in pI was not due to variations in sialic acid or phosphate groups. The nature of the biochemical variation is unknown, but may illustrate an additional level of heterogeneity which is superimposed upon the known variations in sialic acid levels.

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