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The Lymphatic Route. IX. Distribution of Recombinant Interferon-α_2 Administered Subcutaneously with Oedematogenic Drugs

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Summary

We have evaluated whether the addition of either bradykinin or histamine favours the lymphatic absorption of human recombinant interferon-α_2 (IFN-α_2) administered by the subcutaneous route. Subcutaneous administration of IFN-α_2 with bradykinin enhances IFN absorption *via* both capillaries and lymphatics, so that either the plasma or lymph areas under the concentration curves (AUC) increase significantly up to 1751±483 and 1319±608 IU/ml/min respectively as compared to the respective AUC values (613±208 and 483±213 IU/ml/min) obtained after IFN injection in normal saline. Since the lymph AUC/plasma AUC ratios remain unaltered, there is no preferential lymphatic absorption of IFN-α_2 after bradykinin administration. Dual-label experiments, ^{125}I-IFN-α_2 in saline and ^{131}I-IFN-α_2 in saline containing 200 μg histamine were injected subcutaneously into the left and into the right shank of the same animal, gave similar results. The kinetics of ^{125}I and ^{131}I acid-soluble radioactivity confirm that histamine favours both plasmatic and lymphatic absorption.

Key Words

Interferon – Lymphatic absorption – Bradykinin – Histamine

Introduction

Several pharmacokinetic studies have been carried out in patients and animals (Bocci 1987) after intravenous, intramuscular, and subcutaneous (s.c.) administration of cytokines. These routes of administration lead to considerable dilution in body fluids (Bocci 1982), conspicuous renal filtration with tubular catabolism (Bocci *et al.* 1983) and binding to endothelial and parenchymatous cells, which result in toxic effects (Scott 1984, Rosenberg *et al.* 1987, West *et al.* 1987). If cytokines were absorbed preferentially through the lymphatics, they would interact almost exclusively with immunocompetent effector cells abundantly present in lymph and lymph nodes. If less cytokines reached the plasma, toxic effects, which are dose- and plasma-level dependent, would be minimized (Scott 1984). Because the direct intralymphatic route is not practical for prolonged and repeated administration, we sought to overcome this difficulty by s.c. multiple injections of cytokines dissolved in a hyperoncotic human albumin solution (Bocci *et al.* 1988a,b, 1990), thus increasing the interstitial fluid pressure and favouring the absorption of solutes *via* the lymphatics. The absorption of cytokines from the subcutis is variable and depends on their chemical composition, on the chemical interactions with the interstitial phase, on the volume and colloid osmotic pressure of the injected solution, on the location of the injected site and on the activity of the underlying muscles (Bocci 1984). Substances such as histamine and bradykinin are well-known local compounds produced during inflammation and responsible for local vasodilatation, increase of vascular permeability and oedema (Owen 1987, Salmon and Higgs 1987). In this study we have tried, in the rabbit, to evaluate whether the addition of either bradykinin or histamine favours the lymphatic absorption of human recombinant interferon-α_2 (IFN-α_2) administered *via* the s.c. route and whether this approach is more advantageous than the addition of human albumin.

Methods

IFN-α_2 preparation and labelling. Human recombinant interferon-α_2 (Intron-A, Schering-Plough, Milano, Italy) with a specific activity of $2x10^8$ IU/mg proteins was used throughout the experiments. Just before the injection, either bradykinin (Clinalfa, Läufelfingen, Switzerland) or histamine (IBIS, Firenze, Italy) were added at final concentrations of either 50 or 200 μg/ml, respectively. Labelling was carried out as described by Mogensen and Uzé (1986) using either carrier-free ^{131}I or ^{125}I (Amersham, Little Chalfont, England). Using this method, the metabolic behaviour of the labelled IFN was found to be indistinguishable from that of cold IFN (Bocci *et al.* 1983). After iodination, antiviral activity of IFN was practically unchanged and the trichloroacetic acid-soluble radioactivity was no more than 2 %. Radioactivity was counted in a well counter (Clinigamma single, LKB, Bromma, Sweden) that was particularly useful in differentiating of ^{125}I from ^{131}I radioactivity when both were injected simultaneously into the same animal.

Animals. New Zealand male rabbits (2.5-2.8 kg) were kept under conventional conditions for 2 weeks and appeared to be healthy before the experiments. In order to facilitate the localization of the cisterna chyli, the animals were fasted for 2 days before the experiment (water *ad libitum*), except for about 50 g of pellets soaked in olive oil, given the night before the experiment. To ascertain the local oedematogenic effect of bradykinin, three rabbits, after abdomen shaving and depilation, received an s.c. injection into four sites with either 0.2 ml saline (right side) or 0.2 ml of saline containing 5, 10, 15, or 20 micrograms of bradykinin (left side). Immediately thereafter, 2 ml of Evans blue (1 mg/ml saline) were injected intravenously. In order to evaluate local staining due to the extravasation of Evans blue bound to albumin, photographs were taken every minute during the following 15 min and then after the next 30, 45, 60, 120, 180, 240 and 300 min.

Surgery. Rabbits were anaesthetized with 30 mg/kg body weight of pentobarbital sodium (Nembutal, Serva, Heidelberg, Germany) dissolved in saline and injected into the left marginal vein. The right marginal vein was cannulated and a peristaltic pump (LKB, Bromma, Sweden) provided a constant infusion of about 7 ml/h of Emagel (Behringwerke, Marburg/Lahn, Germany) which contained 4 mg/ml piperacillin (Avocin, Lederle, Wayne, USA) and 4 mg/ml pentobarbital sodium. Piperacillin was used in order to reduce bacterial growth after laparatomy. After a midventral laparatomy, the cisterna chyli was exposed and cannulated as indicated by Jakab *et al.* (1980) except that the polythene cannula (ID 0.58 mm) was kept in place with Histoacryl (Braun, Melsungen, Germany). The jugular veins were exposed and, at predetermined times, blood was withdrawn by means of disposable insulin-type syringes. After the surgery, the animals were maintained sedated with Nembutal added to Emagel as described above, with their hind legs in an extended position. Lymph flowed continuously and was collected in heparin hourly for 10 h and blood was collected in heparinized tubes at predetermined times. Because the thoracic duct was not closed, collection of lymph was partial and we could assess only the IFN concentration. Nonetheless, lymph volumes were measured and then lymph and blood samples were centrifuged for 10 min at 2000 x g and at 4 °C. Cell-free lymph and plasma were stored at −20 °C until IFN and radioactivity were determined.

Administration of IFN. In experiments with bradykinin a single dose of 10^7 units of IFN-α_2 in 1 ml of either saline (control) or in 1 ml saline containing 50 μg bradykinin were administered subcutaneously in three sites of the shanks (about 15 μg *per site*), after having shaved and depilated the skin the day before. For the evaluation of the role of histamine, rabbits received orally 0.5 ml of 5 mg/ml cold NaI solution, 12 h before the experiment. Thereafter, they received a s.c. injection simultaneously with 3 μCi of ^{125}I-IFN-α_2 in saline in the left shank and 3 μCi of ^{131}I-IFN-α_2 in saline containing histamine in the right shank. Acid-soluble radioactivity was expressed as a percentage of total radioactivity after precipitation with trichloroacetic acid at a final concentration of 10 % in the presence of cold NaI. Protein-bound radioactivity was obtained by substracting acid-soluble radioactivity from total radioactivity and expressed as cpm/ml. The increase of acid-soluble radioactivity after administration of radiolabelled IFN is due to an increased catabolism of the protein.

IFN determination. IFN titers were determined using the cytopathic effect reduction assay (Armstrong 1981) using WISH cells and Vesicular Stomatitis Virus. All samples were assayed at least twice in triplicate. The assays were always made employing the N.I.H. human IFNα international preparation (reference n° Ga 23-902-530). All titres were expressed in international units per milliliter (IU/ml).

Data analysis. All the data are expressed as mean ± standard deviation (S.D.). The IFN-α_2 distribution and kinetic patterns depending on the type of administration and drugs were evaluated comparing the resulting area under the plasma or lymph concentration curve (AUC) and peak IFN concentration (C_{max}). The AUC and C_{max} of IFN-α_2 in plasma and lymph (either IU/ml or cpm/ml) were obtained fitting the data by the program SAAM 29 (Berman and Weiss 1977). The calculation was performed using the defined integral

$$\int_A^B f(x)dx$$

were A-B is the time interval during which the AUC was calculated corresponding to the first and last sampling point. The resulting values were then divided by the total sampling time in order to obtain the concentration as a function of the experimental time. Rather than comparing the lymph/plasma ratio at individual times, we preferred to use the lymph AUC/plasma AUC ratio, as it gave an overall estimate of the absorption pattern and distribution.

Statistical analyses were performed using Student's t-test, with $p<0.05$ (two-tailed) as the minimal level of significance.

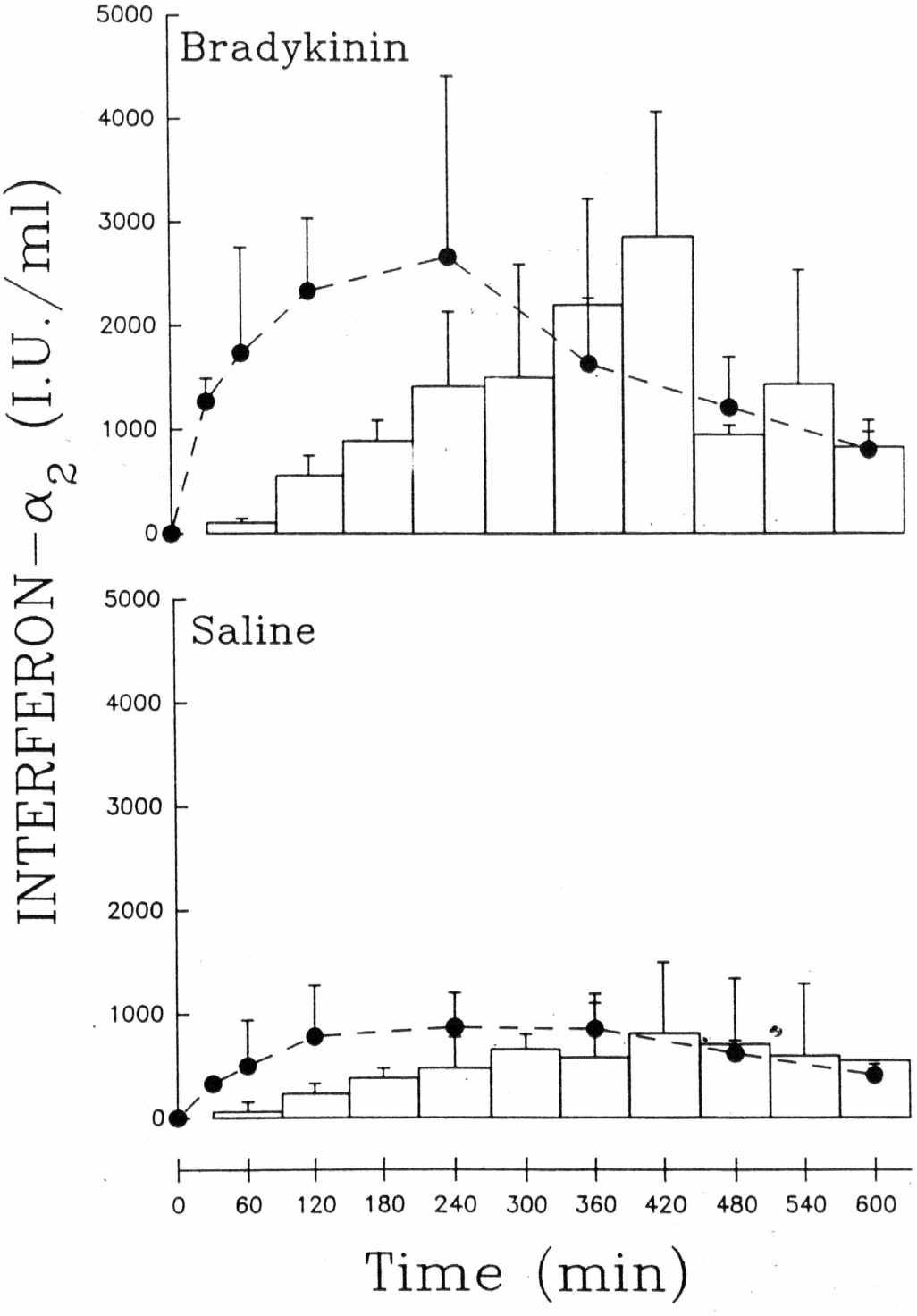

Fig. 1
Distribution of human recombinant IFN-α_2 in rabbit lymph (columns) and plasma (full dots) after s.c. injection of 10^7 units IFN in saline (bottom) or in saline containing 50 μg bradykinin (top). Values are expressed as means ± S.D. (n = 6).

Results

The oedematogenic effect of bradykinin is so rapid that already 1 min after intravenous injection of Evans blue we noticed initial staining at the site of the s.c. injection of bradykinin, reaching maximal intensity 15 min later. The effect of bradykinin is dose-dependent and it is maximal at a concentration as low as 15 μg per site (data not shown). Neither staining nor oedema was visible at the sites injected with saline.

After s.c. administration in saline, IFN-α_2 was absorbed by both blood and lymphatic capillaries, the plasma and lymph AUC being of 613±208 IU/ml/min and 483±216 IU/ml/min, respectively. Thus, besides some small differences in the lymph and plasma C_{max}, the lymph AUC/plasma AUC ratios were about 0.78±0.16 indicating no preferential absorption through the lymphatics (Fig. 1, bottom panel, Fig. 2).

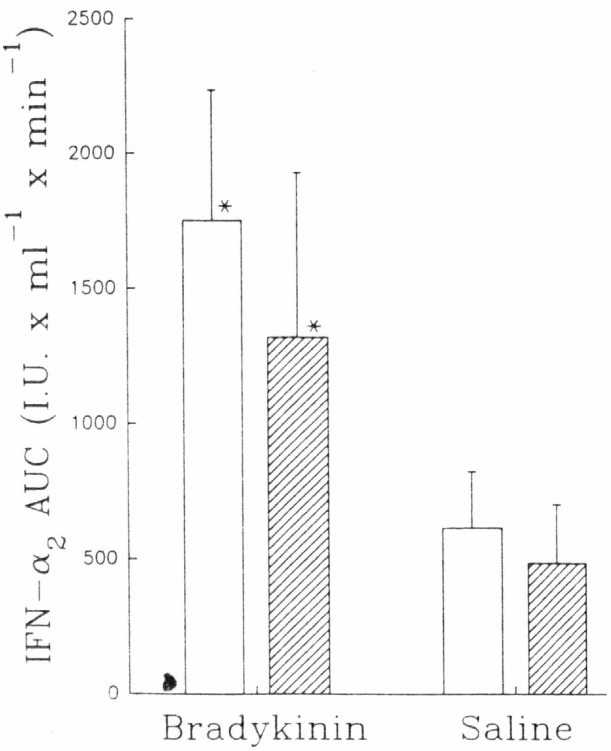

Fig. 2
Plasma (empty columns) and lymph (hatched columns) areas under the curve (AUC) obtained after s.c. injection of 10^7 units of IFN-α_2 in saline or in saline containing 50 μg bradykinin. Values are expressed as means ± S.D. (n = 6). *P < 0.05 in respect to AUC values obtained after IFN injection in saline.

The addition of bradykinin to the same dose of IFN-α_2 markedly enhanced IFN absorption *via* both blood and lymphatic vessels (Fig. 1, top panel), so that IFN concentrations in either plasma or lymph were almost doubled, the C_{max} values being 2668±1749 and 2861±1210 IU/ml, respectively (Fig. 1, top panel). Moreover, both the plasma and the lymph AUC increased significantly ($p<0.05$) up to 1751±483 and 1319±608 IU/ml/min (Fig. 2). The lymph AUC/plasma AUC ratios increased up to 0.9±0.7, but these values were not statistically significant in respect to those obtained after IFN injection in normal saline. The s.c. administration of IFN-α_2 with bradykinin did not cause a noticeable change of lymphatic flow that remained almost constant at a rate of 3.5±1.5 ml/h.

In order to overcome a considerable interindividual variability, the histamine oedematogenic effect was evaluated using the approach of injecting IFN-α_2 labelled with either ^{125}I (saline) or ^{131}I (histamine) into the same animal on the left and on the right shank, respectively. Fig. 3 shows the lymph and plasma protein-bound radioactivity after a typical dual-label experiment carried out in three rabbits. It can be noted that the lymphatic levels of ^{131}I-IFN-α_2 (Fig. 3, top panel) injected with histamine were much higher than levels of ^{125}I-IFN-α_2 injected with saline alone (Fig. 3, bottom panel), suggesting that histamine favoured absorption of IFN from the subcutis. Moreover, the plasmatic AUC were 606±70 cpm/ml/min after IFN s.c. injection in saline and increased significantly ($p<0.05$) up to 1755±138 cpm/ml/min after IFN injection with histamine. Although the data of the lymph AUC could not be statistically evaluated because of the lack of some lymph samplings in one rabbit, they represent a biologically noticeable difference obtained in the same animal. The distribution pattern of the ^{125}I acid-soluble radioactivity was interesting. It was very high in the plasma and lymph samples harvested during the first hour, it decreased during the next three hours and it increased again towards to the end of the experiment (Fig. 4). On the other hand, ^{131}I acid-soluble radioactivity was negligible in the first samples and continuously increased during the experiment (Fig. 4).

Discussion

It has been postulated (Bocci 1984) that, when IFN is used as an immunoenhancer, it should mainly interact with lymphoid cells and it should therefore be administered *via* the lymphatic route. There are several reasons for justifying the preference of this route. Firstly, concentrations of lymphoid cells are 15 to 1000 fold greater in the lymph and lymph nodes than in the plasma, and an increased IFN concentration in the lymph pool should augment the immune response with minimal toxicity. Secondly, IFN causes an enlargement of lymph-nodes and enhances cell activation (Hein and Supersaxo 1988). Thirdly, the release of suppressive components such as prostaglandin E2 and transforming growth factor β1, both potent inhibitors of cell-mediated immunity, should be much higher

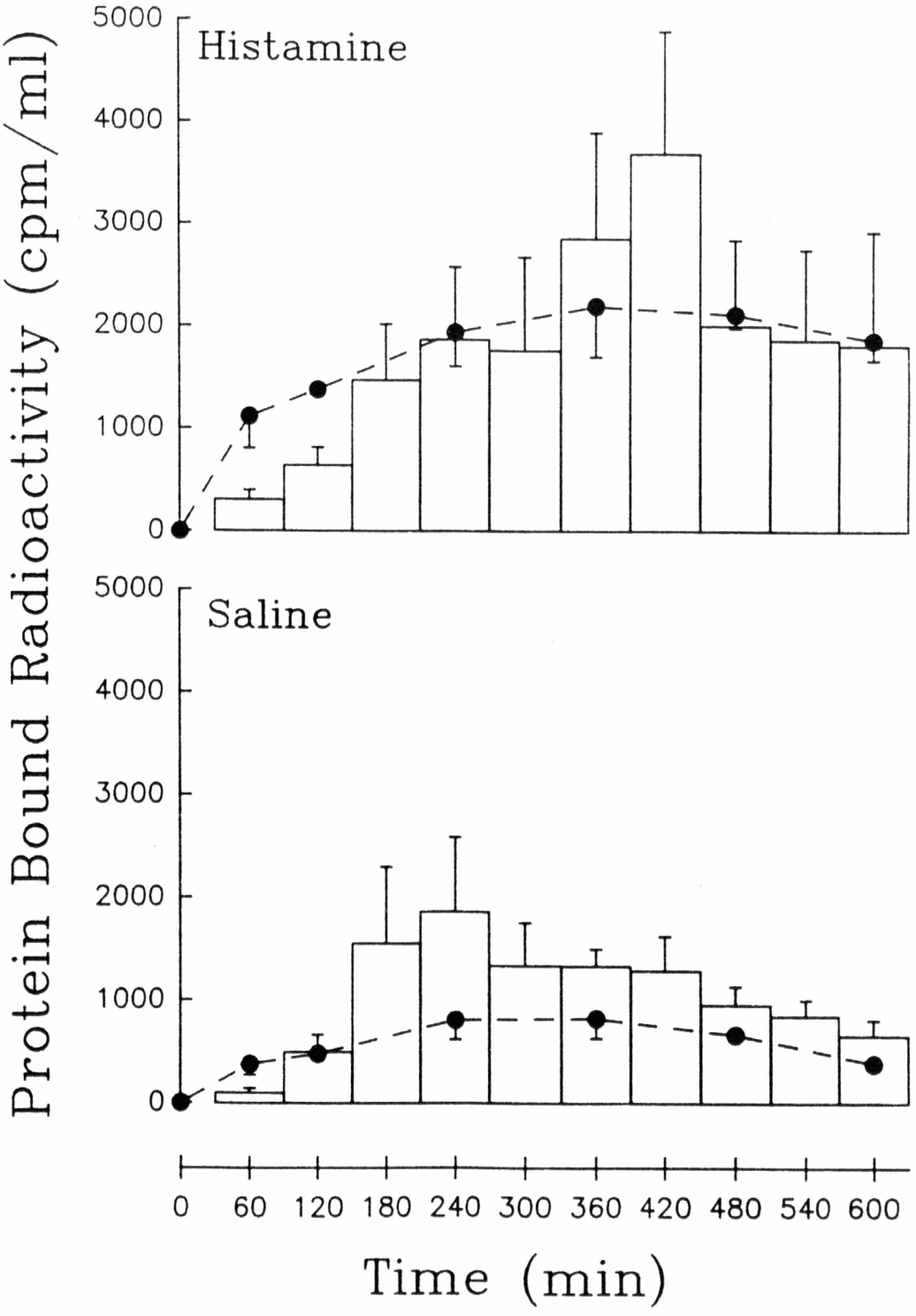

Fig. 3
Distribution of human recombinant IFN-α_2, (expressed as protein-bound radioactivity) in rabbit lymph (columns) and plasma (full dots) after s.c. injection of 3 μCi of either ^{125}I-IFN-α_2 in saline in the left shank (bottom) or ^{131}I-IFN-α_2 in saline containing 200 μg histamine in the right shank (top). Values are expressed as means ± S.D. (n=3).

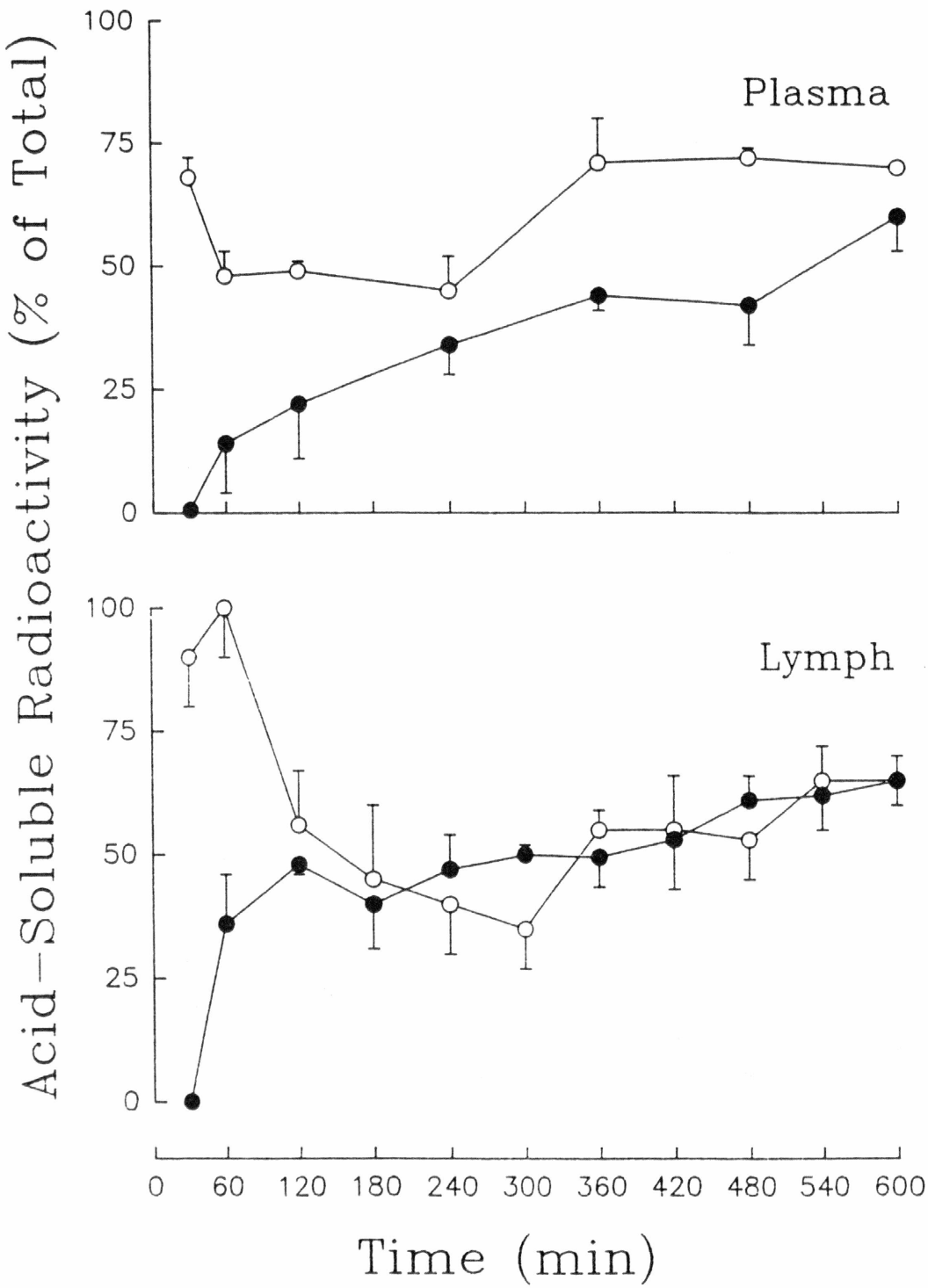

Fig. 4
Acid-soluble radioactivity in rabbit lymph and plasma after s.c. injection of 3 μCi of either ^{125}I-IFN-α_2 in saline (open circles) in the left shank or ^{131}I-IFN-α_2 in saline containing 200 μg histamine (full dots) in the right shank. Values are expressed as means ± S.D. (n=3).

in the blood than in the lymph owing to the presence of monocytes, platelets and neutrophils. Moreover, owing to the nonselectivity of IFN in the blood, it binds to parenchymal cells causing adverse effects.

The intralymphatic infusion of IFN directly into a lymphatic duct with the aid of a pump is possible but is not practical for prolonged treatment. For this reason, we have suggested the indirect method consisting of the s.c. administration of IFN in a hyperoncotic (20 %) human albumin solution. Animal studies (Bocci *et al.* 1988a,b) as well as preliminary evaluation of the pharmacokinetics of interleukin-2 in patients (Gramatzki *et al.* 1986) show that an increased lymphatic absorption of cytokines in the presence of albumin occurs. However, the increase is not dramatic and we felt compelled to undertake the present study to evaluate whether other oedematogenic agents such as prostaglandins, the platelet activating factor, histamine and bradykinin are more effective than albumin. However, in this preclinical phase we have limited the investigation to the latter two drugs because they are practically painless after s.c. injection. It is well known that bradykinin and histamine exert inflammatory activities and produce oedema by increasing protein leakage from the vasculature to the interstitium, the macromolecular leakage occurring primarily from the small venules or the venular ends of capillaries (Miller and Sims 1986). Moreover, bradykinin induces interleukin-6 and prostaglandin production that may have further modulatory effects (Neppl *et al.* 1991, Vandekerckhove *et al.* 1991, Lerner and Modéer 1991). Our results demonstrate that both bradykinin and histamine are unable to enhance the lymph AUC/plasma AUC ratio after s.c. IFN injection significantly, indicating that IFN-α_2 administered together with bradykinin is not preferentially absorbed *via* lymphatic capillaries. On the other hand, these drugs significantly increase both the lymph and plasma AUC as compared to those obtained after IFN administration in saline, clearly indicating that IFN absorption is enhanced from the injection sites. This conclusion is also supported by the observation of the different kinetics of ^{125}I and ^{131}I acid-soluble radioactivities in dual-label experiments probably caused by the different time of IFN permanence in the subcutis. In fact, after the injection of ^{131}I-IFN-α_2 with saline containing histamine, the ^{131}I-acid-soluble radioactivity was very low in the first 30 min and increased continuously during the 10 hours of the experiment. In such a case histamine, which increases both plasmatic and lymphatic IFN absorption, reduces IFN residence time in the subcutis. Alternatively, after the injection of ^{125}I-IFN-α_2 in saline alone, IFN remains in the subcutis for a longer time and local proteolysis may be responsible for the high initial ^{125}I acid-soluble radioactivity observed in lymph and plasma samples.

In conclusion, histamine and bradykinin appear to facilitate the bioavailability of interferon after s.c. administration but do not allow a selective increase of its lymphatic absorption that would be highly desirable for improving the therapeutic index of interferon.

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References

ARMSTRONG J.A.: Cytopathic effect inhibition assays for interferon: microculture plate assay. In: *Methods of Enzymology*. S. PESTKA (ed.) Academic Press, New York, 1981, pp. 381–387.

BERMAN M., WEISS M.F.: SAAM 27. NIH, Bethesda, Maryland, 1977.

BOCCI V.: Catabolism and toxicity of interferons. *Drugs Exp. Clin. Res.* **8**: 683–689, 1982.

BOCCI V.: Evaluation of routes of administration of interferon in cancer: a review and a proposal. *Cancer Drug Del.* **1**: 337–351, 1984.

BOCCI V.: Metabolism of protein anticancer agents. *Pharmacol. Ther.* **34**: 1–49, 1987.

BOCCI V., MOGENSEN K.E., MUSCETTOLA M., PACINI A., PAULESU L., PESSINA G.P., SKIFTAS S.: Degradation of human ^{125}I-interferon alpha by isolated perfused rabbit kidney and liver. *J. Lab. Clin. Med.* **101**: 857–863, 1983.

BOCCI V., PESSINA G.P., PAULESU L., MUSCETTOLA M., VALERI A.: The lymphatic route. V. Distribution of human natural interferon-β in rabbit plasma and lymph. *J. Interferon Res.* **8**: 633–640, 1988a.

BOCCI V., PESSINA G.P., PAULESU L., NICOLETTI C.: The lymphatic route. VI. Distribution of recombinant interferon-α_2 in rabbit and pig plasma and lymph. *J. Biol. Resp. Modif.* **7**: 390–400, 1988b.

BOCCI V., PESSINA G.P., NICOLETTI C., PAULESU L.: The lymphatic route. VII. Distribution of recombinant human interleukin-2 in rabbit plasma and lymph. *J. Biol. Regul. Homeost. Agents* **4**: 25–29, 1990.

GRAMATZKI M., NÜSSLEIN H., BURMESTER G.R., RÖDL W., HEYDER N., GROTE W., MONNER D.A., MÜHLRADT P.F., KALDEN J.R.: Intralymphatic interleukin-2 treatment in patients with acquired immunodeficiency syndrome: preliminary experience in three cases. *Immunobiology* **172:** 438–447, 1986.

HEIN W.R., SUPERSAXO A.: Effect of interferon-alpha-2a on the output of recirculating lymphocytes from single lymph nodes. *Immunology* **64:** 469–474, 1988.

JAKAB F., SUGAR I., SZABO G.: Cannulation of the cervical thoracic duct in the rat. *Lymphology* **13:** 184–185, 1980.

LERNER U.H., MODÉER T.: Bradykinin B1 and B2 receptor agonists synergistically potentiate interleukin-1-induced prostaglandin biosynthesis in human gingival fibroblasts. *Inflammation* **15:** 427–436, 1991.

MILLER F.N., SIMS D.E.: Contractile elements in the regulation of macromolecular permeability. *Fed. Proc.* **45:** 84–88, 1986.

MOGENSEN E.K., UZÉ G.: Radioiodination of human alpha interferons by the chloramine T method. *Meth. Enzymol.* **119:** 267–276, 1986.

NEPPL H., NEUHOF H., AFFLERBACH F., LLACH PUIG NEPPL J., BERGH FER A.: Bradykinin-induced oedema formation proceeds from B_2 receptor stimulation and is potentiated by concomitantly released prostaglandins. *Acta Physiol. Scand.* **142:** 141–147, 1991.

OWEN D.A.A.: Inflammation-histamine and 5-hydroxytryptamine. *Br. Med. Bull.* **43:** 256–269, 1987.

ROSENBERG S.A., LOTZE M.T., MUUL L.M., CHANG A.E., AVIS F.P., LEITMAN S., LINEHAN W.M., ROBERTSON C.N., LEE R.E., RUBIN J.T., SEIPP C.A., SIMPSON C.G., WHITE D.E.: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *New Engl. J. Med.* **316:** 889–897, 1987.

SALMON J.A., HIGGS G.A.: Prostaglandins and leukotrienes as inflammatory mediators. *Br. Med. Bull.* **43:** 285–296, 1987.

SCOTT G.M.: The toxic effects of interferon in man. In: *Interferon*. I. GRESSER (ed.) Academic Press, New York, 1984, pp. 85–114.

VANDEKERCKHOVE F., OPDENAKKER G., VAN RANST M., LENAERTS J.P., PUT W., BILLIAU A., VAN DAMME J.: Bradykinin induces interleukin-6 and synergizes with interleukin-1. *Lymphokine Cytokine Res.* **10:** 285–289, 1991.

WEST W.H., TAUER K.W., YANNELLI J.R., MARSHALL G.D., ORR D.W., THURMAN G.B., OLDHAM R.K.: Constant infusion of recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. *New Engl. J. Med.* **316:** 898–905, 1987.

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