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REDUCTION IN ARM SWELLING AND CHANGES IN PROTEIN COMPONENTS OF LYMPHEDEMA FLUID AFTER INTRAARTERIAL INJECTION OF AUTOLOGOUS LYMPHOCYTES

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ABSTRACT

The intraarterial arm injection of freshly isolated autologous lymphocytes to a patient with upper extremity secondary lymphedema brought about a rapid and remarkable reduction in arm swelling. The protein components in the edema fluid were analyzed by two-dimensional electrophoresis before and after lymphocyte injection. We observed the appearance of a novel protein spot, with an isoelectric point of 6.5, in an electropherogram as early as 30 minutes after the lymphocyte injection. Immunoblotting using antibody against human total serum proteins suggested that the novel protein was not derived from the serum. Because incubation in vitro *of the lymphedema fluid with the isolated lymphocytes produced a new protein spot, corresponding to the novel protein observed* in vivo, *we suspect that the novel protein originated from limited hydrolysis of a unique protein present in the arm edema fluid. Significance of the novel protein and the role of limited proteolysis after lymphocyte injection in the management of lymphedema are examined.*

Peripheral lymphedema commonly develops after operations such as radical mastectomy and radical hysterectomy. Approximately 10 years ago, we injected a suspension of homologous intraarterially to a patient with disseminated cancer of the rectum three years after abdominal-perineal resection in an attempt to forestall advancement of the rectal malignancy. Although the lymphocyte injection did not have the desired anti-tumor effect, we unexpectedly observed a dramatic reduction of leg lymphedema. Subsequently, regression of peripheral lymphedema after intraarterial injection of homologous lymphocytes was confirmed in several other patients who developed limb swelling after radical operations for a variety of malignancies (1). After these experiences, we administered autologous lymphocytes intraarterially to a number of patients as part of a standard treatment for lymphedema of extremities in conjunction with other non-operative therapy. More recently we found that injection of *in vitro* cultured lymphocytes produced a similar beneficial effect to that of freshly prepared autologous lymphocytes on lymphedema (2). Thus far, however, the mechanism of the efficacy of lymphocyte injection therapy has remained unknown.

In the present study, lymphedema fluid was collected from a patient who had shown remarkable regression of arm swelling to repeated administrations of cultured lymphocytes intraarterially and the protein components of the edema fluid were analyzed by two-dimensional electrophoresis. Not only was a novel protein spot recognized on the gel shortly after lymphocyte injection, but incubation of the edema fluid with the isolated

lymphocytes *in vitro* induced the appearance of a similar novel protein spot. The significance of these findings is examined in terms of the mechanism of action of the injected lymphocytes.

MATERIALS AND METHODS

The patient in this study is a 47-year-old woman with lymphedema of the left arm after a radical mastectomy. She had previously shown reduction of arm swelling to repeated injections of freshly prepared autologous lymphocytes, and was now admitted to the hospital for another course of intraarterial injection therapy.

The lymphocyte fraction was isolated from the patient's blood using a blood cell separator (IBM 2997). A suspension (100ml) of lymphocytes ($2x10^9$ cells) in the serum was injected into the proximal portion of the subclavian artery of the patient's affected arm. Left arm edema fluid was collected by puncturing the back of her swollen hand with a side-holed 22-gauge needle before and after the lymphocyte injection at the prescribed times.

The protein concentrations in the serum and edema fluid were determined by the method of Lowry *et al* (3).

The proteins in the serum and edema fluid were separated by the technique of two-dimensional polyacrylamide gel electrophoresis described by Manabe *et al* (4) with some modifications. The technique employed isoelectric focusing in the first dimension and gradient gel electrophoresis in the second dimension. The serum or edema fluid (10µl) containing 40% (w/v) sucrose was subjected to the first dimensional isoelectric focusing by applying a constant current of 2 mA/tube for 30 minutes and then a constant voltage of 200V for 2 hours at 4°C. The second dimensional electrophoresis was carried out at a constant current of 20 mA/tube per slab for 1 hour at 4°C. After the electrophoresis, gels were silver-stained (5).

Immunoblot analysis of serum and edema fluid was carried out by staining nitrocellulose paper after electrophoretic transfer from the slab gel (6) with antibody against human serum proteins (DAKO Corporation) and with anti-rabbit IgG-peroxide conjugate (Cappel Laboratories, Inc.).

For the preparation of the soluble fraction of lymphocytes, the freshly isolated lymphocytes were homogenized in phosphate-buffered saline with a Polytron homogenizer. The homogenate was centrifuged at 104,000xg for 1 hour and the supernatant was used for two-dimensional electrophoretic analysis.

For the *in vitro* incubation of the edema fluid with the isolated lymphocytes, the edema fluid (0.5ml) was incubated with the lymphocytes ($2x10^7$ cells) at 37°C for 30 minutes. The incubated fluid, after sedimentation of lymphocytes, was subjected to two-dimensional electrophoretic analysis.

RESULTS

As early as several hours after the intra-arterial injection of freshly isolated autologous lymphocytes, the patient demonstrated a rapid and remarkable reduction in the lymphedematous swelling of the left hand. The swelling continued to regress for two days and the improved state lasted for several weeks. On the third day after lymphocyte injection, the reduction of arm swelling was so marked that collection of edema fluid was virtually impossible *(Fig. 1)*.

Arm edema fluid was collected at 30 minutes and 4 hours after lymphocyte injection, and the protein contents of the samples were compared with an edema fluid sample taken before the injection. All three samples showed a protein level of 15mg/ml, indicating that lymphocyte injection did not change the concentration of the total proteins in the edema fluid. However, when the edema fluid was analyzed by two-dimensional electrophoresis, a novel protein spot appeared in the sample collected at 30 minutes after lymphocyte injection *(Fig. 2B)*. The novel protein possessed an isoelectric point of 6.5. Its molecular weight could not be determined because the second dimensional electro-

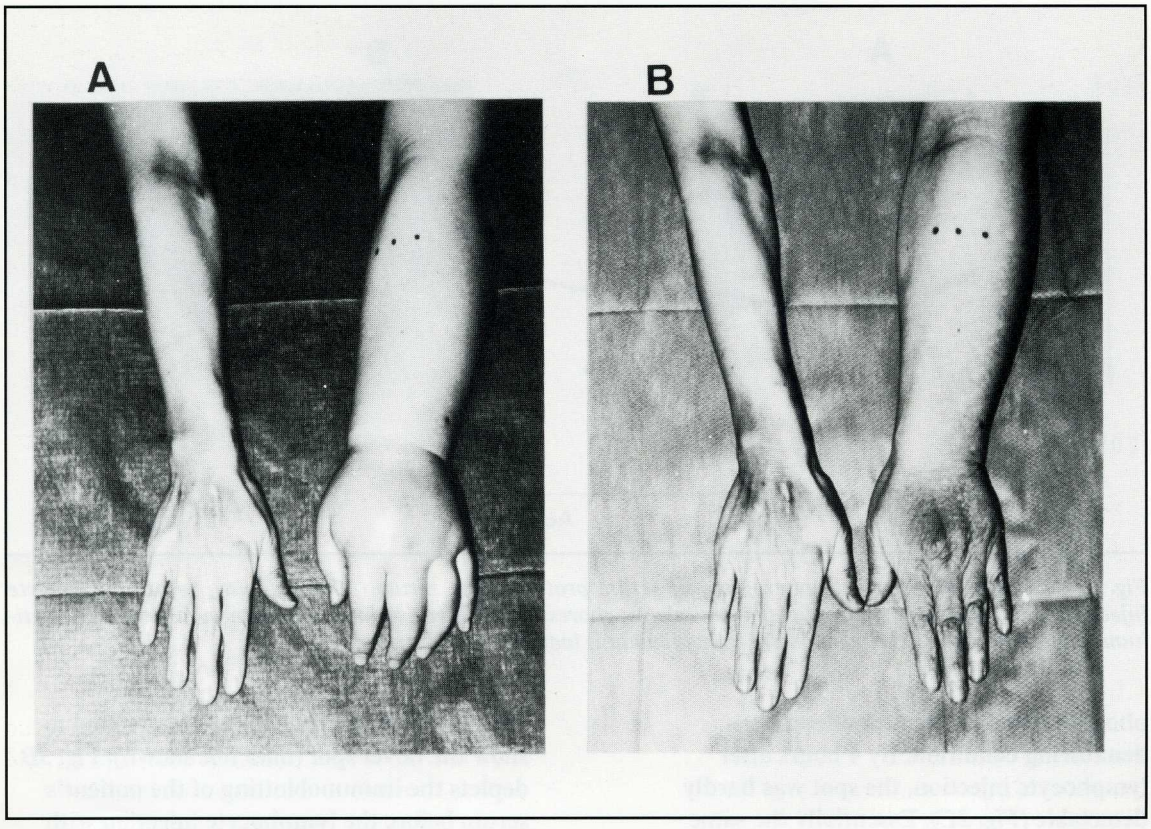

Fig. 1. A 47-year-old woman with lymphedema of left arm before (A) and 3 days after (B) intraarterial lymphocyte injection.

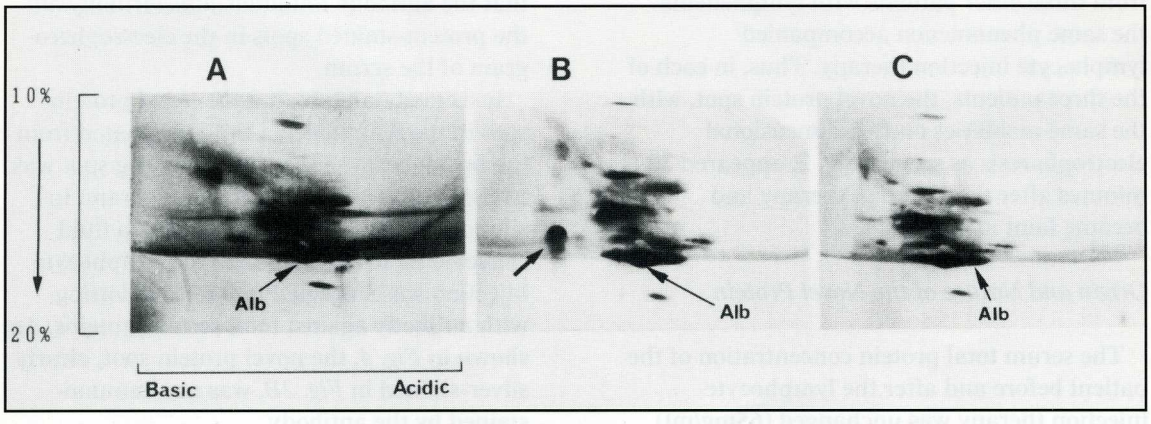

Fig. 2. Two-dimensional electropherogram of the arm lymphedema fluid. The edema fluid, containing 75μg protein, was subjected to two-dimensional electrophoresis. The first dimensional isoelectric focusing was performed on a pH 4-8 gradient and the second on a 10-20% linear gradient acrylamide gel. After electrophoresis, the gel was silver-stained. (A) Before lymphocyte injection. (B) Thirty minutes after lymphocyte injection. (C) Four hours after lymphocyte injection. The short, thick arrow shows the location of the novel protein spot and the longer, thin arrow shows the site of albumin.

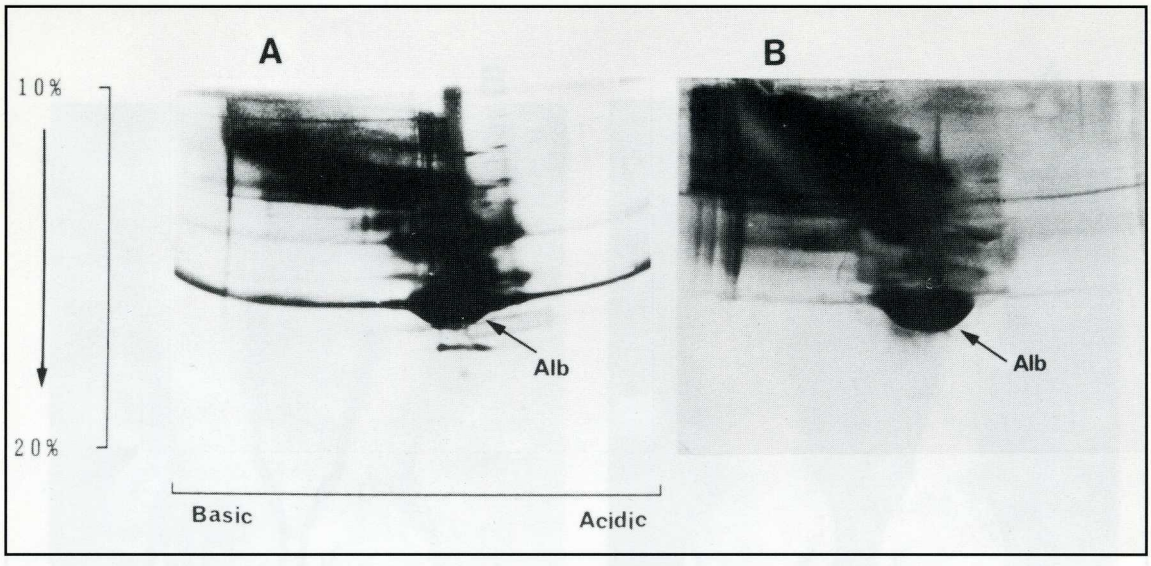

Fig. 3. Two-dimensional electropherogram of serum proteins. The serum (75μg protein), before lymphocyte injection, was subjected to two-dimensional electrophoresis. (A) Silver-stained. (B) Immunoblotted with anti-human serum proteins. The position of serum albumin is indicated by the arrows.

phoresis was carried out under a non-denaturing condition. By 4 hours after lymphocyte injection, the spot was hardly detectable *(Fig. 2C)*. Essentially the same electrophoretic appearances were obtained each time the patient underwent lymphocyte injection therapy. Moreover, in edema fluids from three other patients with lymphedema the same phenomenon accompanied lymphocyte injection therapy. Thus, in each of the three patients, the novel protein spot, with the same mobilities on two-dimensional electrophoresis as seen in *Fig. 2*, appeared 30 minutes after the injection therapy and became faint at 4 hours.

Origin and Nature of the Novel Protein

The serum total protein concentration of the patient before and after the lymphocyte injection therapy was unchanged (65mg/ml). A two-dimensional electropherogram of the patient serum before lymphocyte injection is shown in *Fig. 3A*. There is no protein spot corresponding to the novel protein spot seen in *Fig. 2B*. A serum sample prepared 30 minutes after the lymphocyte injection also failed to show the novel spot (data not shown). *Fig. 3B* depicts the immunoblotting of the patient's serum before the lymphocyte injection with the antibody against human serum proteins. The appearance is essentially the same as the protein-stained picture of *Fig. 3A*, suggesting that the antibody immunostains virtually all the protein-stained spots in the electropherogram of the serum.

It seemed unlikely that the novel protein spot in the lymphedema fluid originated from the serum because the corresponding spot was not seen in the serum electropherogram. In support of the conclusion, the edema fluid collected at 30 minutes after the lymphocyte injection was subjected to immunoblotting with antibody against total serum proteins. As shown in *Fig. 4*, the novel protein spot, clearly silver-stained in *Fig. 2B*, was not immunostained by the antibody.

Could the novel protein have been secreted or leaked from the injected lymphocytes? This possibility was checked by examining the two-dimensional electropherogram of the soluble protein fraction of isolated lymphocytes. As

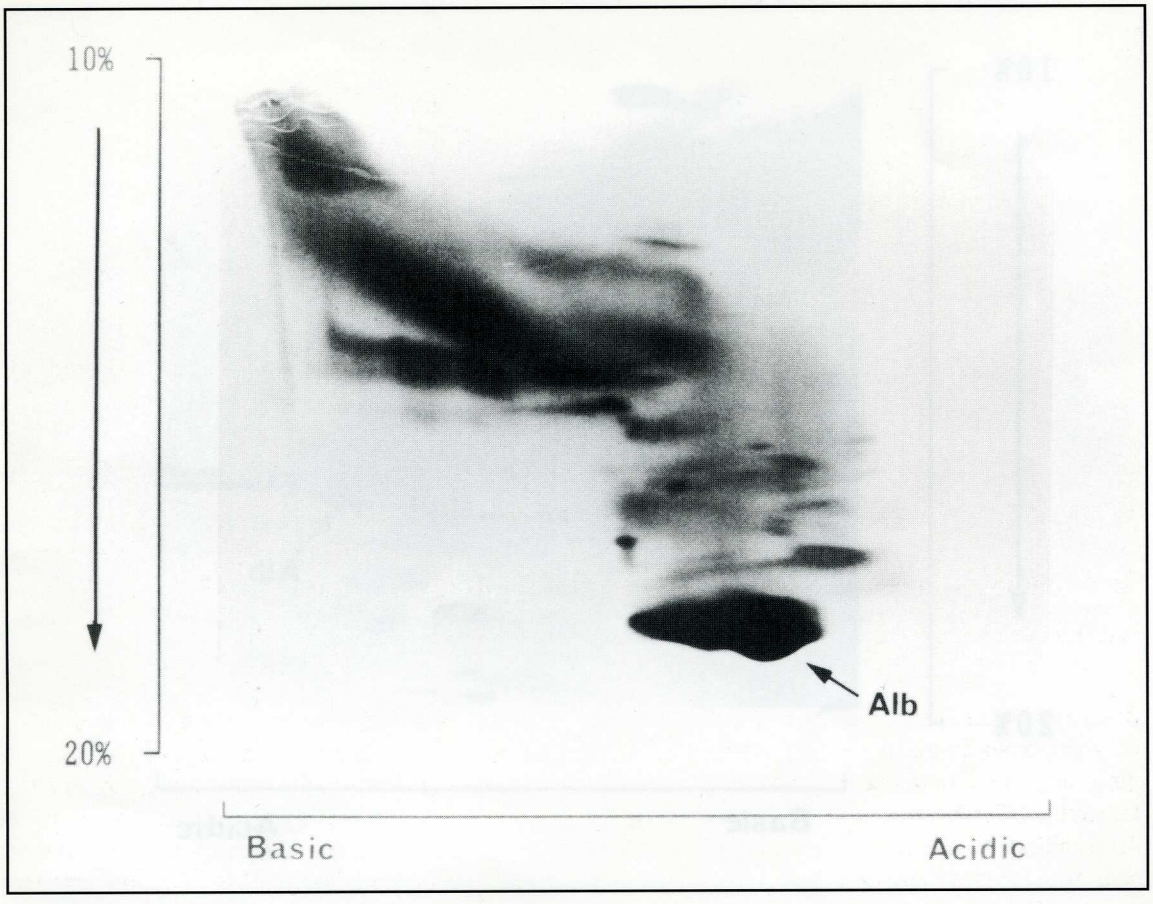

Fig. 4. Immunoblot analysis of the lymphedema fluid, collected at 30 minutes after the lymphocyte injection. The edema fluid (75μg protein) was subjected to two-dimensional electrophoresis and immunoblotted with anti-human serum proteins. The position of albumin is indicated by the arrow.

seen in *Fig. 5*, the silver staining did not display the spot corresponding to the novel protein with an isoelectric point of 6.5.

The foregoing experiments demonstrated that the novel protein appearing in the edema fluid shortly after the lymphocyte injection was derived neither from serum proteins nor from the cytoplasm of injected lymphocytes. The possibility remains that the novel protein represented the product of limited proteolysis of a protein uniquely present in the edema fluid. Perhaps interaction of the edema fluid with the injected lymphocytes stimulated such limited proteolysis. In order to check this possibility, the edema fluid, collected before the lymphocyte injection, was incubated *in vitro* with the isolated lymphocytes and the incubated fluid was analyzed by two-dimensional electrophoresis. The result, depicted in *Fig. 6*, shows the appearance of a new protein spot in the incubated sample. The newly appeared spot had an isoelectric point around 6.5 and was located near the novel protein spot observed in *Fig. 2B*.

DISCUSSION

The intraarterial injection of a suspension of freshly isolated autologous lymphocytes to a patient with secondary lymphedema promoted a rapid and remarkable reduction in swelling of arm lymphedema. Swelling at time of

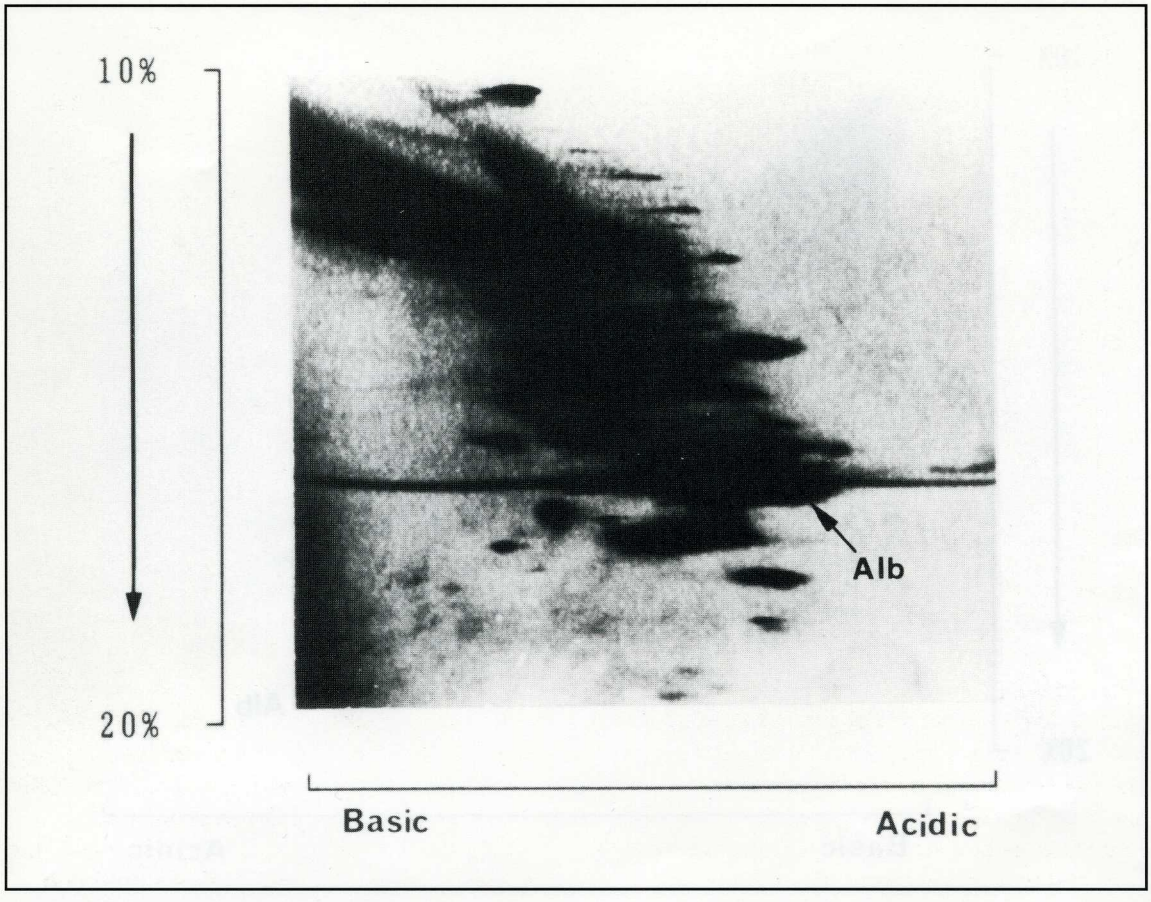

Fig. 5. Two-dimensional electropherogram of lymphocyte soluble proteins. The soluble protein fraction of the isolated lymphocytes (75μg protein) was subjected to two-dimensional electrophoresis and silver-stained.

admission to the hospital was marked and use of lymph drainage massage and elastic bandaging proved overly painful for the patient. Elevation of the swollen arm for several days slightly reduced the edema but the effect was considerably less than after lymphocyte injection therapy.

In the present study, the protein components in the lymphedema fluid were analyzed by two-dimensional electrophoresis before and after the lymphocyte injection. We observed the appearance of a novel protein spot in the electropherogram as early as thirty minutes after lymphocyte injection. The appearance of this unique protein spot was highly reproducible; the same spot was repeatedly detected each time the patient underwent several lymphocyte injection treatments as well as when lymphocyte therapy was administered to three other patients with lymphedema.

The novel protein had an isoelectric point of 6.5 but the electrophoretic device we used did not permit estimation of the molecular weight. Absence of the novel protein by immunoblotting analysis of edema fluid using antibody against total serum protein suggested that the novel protein was not derived from the serum. Moreover, a two-dimensional electrophoretic analysis of the soluble proteins of lymphocytes indicted that the novel protein did not likely originate from the cytoplasm of the injected lymphocytes. On the other hand, when the edema fluid was incubated *in vitro*

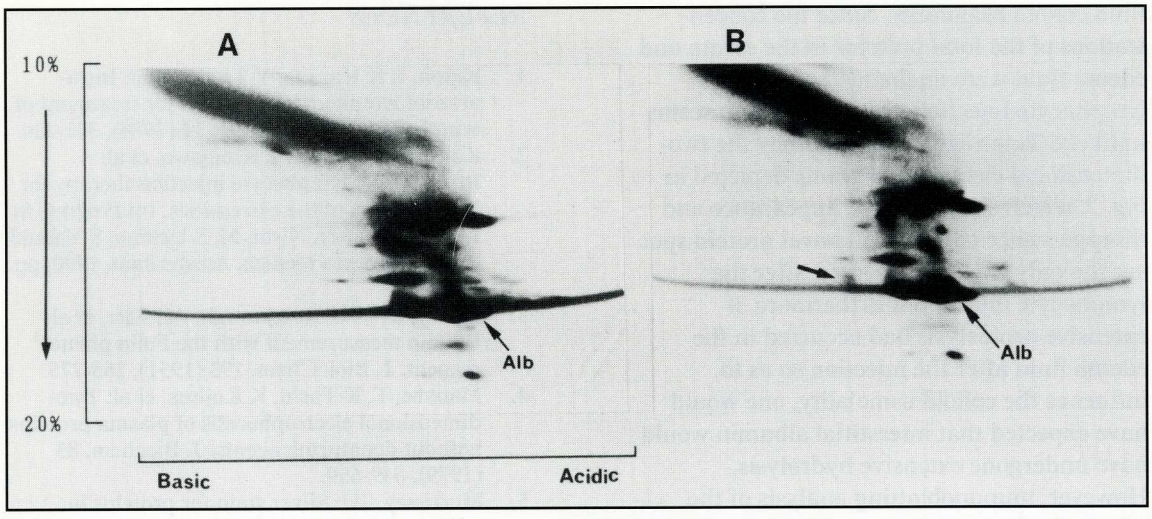

Fig. 6. Two-dimensional electropherogram of the lymphedema fluid incubated in vitro with the isolated lymphocytes. The edema fluid (75μg protein) before and after incubation with lymphocytes was subjected to two-dimensional electrophoresis and silver-stained. (A) Before incubation. (B) Incubated for 30 minutes. The position of a newly appeared protein spot is indicated by the short, thicker arrow and albumin by the thinner, longer arrow.

with the isolated lymphocytes, a new protein spot appeared in the vicinity of the novel protein spot previously detected in the edema fluid *in vivo* after the lymphocyte injection. If the newly-appeared protein spot *in vitro* was in fact the same as the novel protein spot observed *in vivo*, it suggests an origin from limited hydrolysis of a protein present in the edema fluid but absent from the serum. If the novel protein had been derived from hydrolysis of serum proteins, the novel protein spot should have stained positively in the immunoblotting experiment using antibody against the total serum proteins.

There is no direct evidence that the novel protein plays a role in the reduction and swelling of the lymphedematous limb. Nonetheless, the rapid appearance of the novel protein spot after the lymphocyte injection along with decrease in lymphedema suggests that the two phenomena are causally related. Even if the novel protein itself is not directly involved in the therapeutic benefit of the injected lymphocytes, the present study has demonstrated that limited proteolysis occurs in the lymphedema fluid soon after lymphocyte injection. Although the novel protein spot, shown in *Fig. 2*, is the only clearly identifiable spot, we can not exclude the possibility that limited proteolysis produces other unidentified byproducts. If the electrophoretic mobilities of proteolytic byproducts either overlap those of pre-existing protein components of the edema fluid or if their amount is extremely small, they may have escaped detection.

Whatever the significance of the limited proteolysis after the lymphocyte injection therapy, we assume that the injected lymphocytes are responsible for this phenomenon. Although we do not have direct data for selective accumulation of the injected lymphocytes into the edematous interstitial tissue, a recent study in rats has shown the preferential migration of injected lymphocytes into a thermally induced edematous region (7). It is, therefore, possible that chronic lymphedema induces the formation of high endothelial venules through which the injected lymphocytes are rapidly transported into the swollen interstitial tissue.

A number of factors can influence the progression or regression of edema. One possibility is a change in the serum or edema

fluid colloid osmolality. Since the concentrations of the total proteins in the serum and edema fluid were unchanged after the lymphocyte injection, this explanation seems unlikely. This view is supported by the two-dimensional electropherograms depicted in *Fig. 2*, which show that the appearance and disappearance of the small novel protein spot are the only changes observed after the lymphocyte injection. Furthermore, if extensive proteolysis had occurred in the edema fluid after the injection so as to influence the colloid osmolality, one would have expected that interstitial albumin would have undergone extensive hydrolysis. However, immunoblotting analysis of the edema fluid after administration of antibody against human serum albumin failed to show notable degradation of albumin molecules (data not shown).

Perhaps there was a change in regional capillary filtration rate in the edematous arm or a rise in lymph flow. However, since lymph flow in the patient's affected arm was severely restricted as a consequence of radical mastectomy, it seems unlikely that lymph flow could accelerate soon after lymphocyte injection therapy.

Various physiologic substances influence the transcapillary filtration rate. Our results suggest that injected lymphocytes convert, by limited hydrolysis, some proteins in the edema fluid to physiologically active peptides which alter capillary filtration. Whether the observed novel protein performs such a function remains to be determined.

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