Tissue Changes in Chronic Experimental Lymphoedema in Dogs

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Summary

Chronic lymphoedema was experimentally induced in the legs of dogs and studied with the electron microscope, including by quantitation. It was found that some cells (macrophages, fibroblasts and, to a lesser extent, lymphocytes) increased greatly in numbers and relative volumes. Collagen (and fat cells) also greatly increased in relative volume. The lengths of blood vessels and initial lymphatics were much greater in the injured tissue. The numbers of small vesicles and vacuoles rose greatly in both types of vessels. Both also had many open endothelial junctions — although no doubt from different causes. It was concluded that, just as chronic inflammation is probably caused by excessive accumulations of proteins, so chronic lymphoedema is probably a form of chronic inflammation.

Introduction

The fine structural alterations in lymphoedema have been described, qualitatively, by a number of workers (Altorfer and Clodius 1976, Altorfer et al. 1977; Asano 1974; Casley-Smith 1977a, 1980a; Casley-Smith et al. 1974, 1977, 1978a; Cremer et al. 1973, 1974; Huth 1972, 1980; Kalima 1971; Pfleger 1964; Piller 1977; Olszewski 1977; Rusznyak et al. 1967; Veress et al. 1966). It has been suggested (Casley-Smith 1976, 1979a, 1980a; Casley-Smith and Gaffney 1980; Földi and Casley-Smith 1978; Gaffney and Casley-Smith 1980) that lymphoedema is a form of chronic inflammation — especially in its latent and chronic stages. This is based on the facts that very similar pathological alterations are found in the tissues, and Willoughby and Di Rosa's (1970) hypothesis, that the basic cause of all inflammation is altered, tissue proteins. It has recently been shown (Casley-Smith and Gaffney 1980; Gaffney and Casley-Smith 1980) that in rat subcutaneous tissue a simple excess of plasma proteins without lymphatic obstruction is sufficient to produce in 64 days the alterations of chronic inflammation, and — importantly — of chronic lymphoedema. The alterations observed included the presence of many macrophages and fibroblasts, considerable fibrosis, a mild invasion of small lymphocytes (but not blast cells), and an increase in the numbers of blood capillaries and initial lymphatics. The vessels had increased numbers of small vesicles and vacuoles in their endothelium and many open junctions. The lymphatics also had these, and were dilated with much protein in their lumens — here, not because of lymphostasis, but because of excess high-protein load. In effect there was "lymphoedema without lymphostasis"! For this reason, there were no thrombi in the lymphatics, nor were their basement membranes less tenuous, nor were the walls of the lymph collectors as greatly altered as previously reported (Altorfer and Clodius 1976, Altorfer et al. 1977). However, all the other alterations, in tissues and blood vessels, were remarkably similar to those seen in chronic lymphoedema. The alterations in these experiments were largely

Dedicated to Prof. M. Földi on the occasion of his sixtieth birthday.
quantitated. However, no quantitative electron microscopy of lymphoedema, nor, for comparison, of other inflammatory reactions has been carried out so far.

The present work is a quantitative electron microscopic study of chronic lymphoedema in the leg of the dog. Since Clodius (1980) has noted that lymphoedematous tissue has a very high vascularity, and Altorfer et al. (1977) and Casley-Smith et al. (1977) noted some morphological alterations to its endothelium, particular attention was paid to these.

Material and Methods

In four mongrel dogs chronic secondary lymphoedema was produced, three having a type III and one a type II procedure. (Clodius and Altorfer 1977; Clodius, 1977). Their lymphoedematous tissue was biopsied after periods of 3, 11 and 42 months for the type III operations and 12 months for the type II. Control tissue was taken from four normal, similar, animals. These last were used, rather than the unoperated legs, because it has been shown (Matsions 1980) that lymphoedema causes alterations — at least to macrophage numbers — in tissues in quite different regions of the body than just those distal to the operation site. Pieces of subcutaneous tissue, measuring 2 x 2 x 1 mm were isolated on all sides by ligatures to prevent the cut lymphatics from collapsing and immersed in fixative.

The fixative, 4 g/100 ml glutaraldehyde in Millonig’s (1961) buffer, was employed for 1 hour at 4 °C. The blocks were briefly washed and were post-fixed in 2 g/100 ml OsO₄ in the buffer, dehydrated in acetone and embedded in araldite. Sections were stained with lead (pH 1.0) and Uranyl acetate. They were examined in a Siemens Elmiskop I, fitted with a Faraday-cage, so that only 50—60 nm sections were studied (Casley-Smith and Crocker 1975).

Two sections per animal were scanned at a magnification of 7,200 x (checked with a grating replica — 2160 lines/mm, and found to be reproducible to within 0.5%). An average of 25 random fields were observed per dog (after excluding any) with a 7-point grid. This was done in the electron microscope itself, using the binocular viewer — where necessary. The incidences of the points on the various tissue features were recorded, and used for estimating Vv (volumes per unit volume — Weibel 1969). Standard Errors were estimated from the Binomial Distribution. Those falling on cells with visible nuclei were differentiated according to cell-type, using the classification scheme and criteria of Schroeder (Schroeder and Munzel-Pedrazzoli 1973; Schroeder et al. 1973a). Non-nucleated cells were simply recorded as such, with the exception of fat containing cells, which were probably largely macrophages: see results.

All the cell-types of the nucleated-cells, and the vessels, falling in the field (123 µm²) were recorded, using the same criteria, and the Nv’s estimated (numbers per unit area — Weibel 1969). Standard Errors were estimated using the Poissonian Distribution. The Nv values were converted to Nv’s (numbers per unit volume) by dividing them by the caliper diameters of the various cell nuclei (DeHoff and Rhines 1961), which is probably more accurate than the method used by Schroeder (loc. cit.) and is now possible since the caliper diameters have been estimated. The diameters used were: macrophages 7.65, Standard Deviation (S.D.) 0.17 (Crapo and Greeley 1978), monocytes 5.86, S.D. 0.41 (Guix, 1979, pers. com.), fibroblasts and fibrocytes 7.64, S.D. 0.35 (Crapo and Greeley 1978), small lymphocytes 7.0, S.D. 0.5 (Casley-Smith 1979, unpubl.). The Ly’s (lengths per unit volumes) of the vessels were estimated by doubling their Nv’s (Weibel 1969). These are used since it seems more meaningful to talk of the total lengths of a particular type of vessel in a cube of tissue, rather than how often one sees it in a cross-section, but it must be realised that these are total lengths.

The quantitative morphology of the blood vessels was studied in a manner similar to that used by Casley-Smith and Window (1976). At a total photographic magnification of 75,200 x (S.E. 370), 25 random portions of
endothelium, 2 μm long, were studied for each group, and the numbers of small vesicles and vacuoles counted. The former have an upper limit rather less than 100 nm, and their combined distribution is definitely bimodal — Casley-Smith and Window (1976). The numbers of free vesicles and vacuoles deep to each 1 μm2 of endothelium were calculated using the section thicknesses (Casley-Smith and Crocker 1975) and allowing for lost caps (Weibel 1969). We also allowed for the random angles of sectioning, which artefactually increases the apparent cell width (Casley-Smith and Davy 1978). One hundred random small vesicles and 50 vacuoles, per group, had their root mean square (R.M.S.) internal diameters measured, again allowing for random angles of sectioning and resolving power (Casley-Smith and Davy 1978). At 21,000 x (S.E. 110), 100 junctions per group were observed and it was noted if they were open (wider than 30 nm) in whole or in part — neglecting the gaps between any luminal projections (Casley-Smith and Window 1976). It was considered that any junction open along part of its depth was actually open completely in a plain other than that of the section (Casley-Smith and Window 1976; Collan and Kalima 1974). Differences between the various values were tested for significance using the t-distribution.

Results

General observations

The tissues in general showed the usual alterations of lymphoedema (Figs. 1–5, 7–10), with considerable fibrosis (Fig. 1). There was much oedema (Figs. 2–5), with a high protein concentration: 5.8 g/dl (S.E. 0.34) in the lymphoedematous interstitial tissue, 5.9 g/dl (0.29) in its initial lymphatics, 0.72 g/dl (0.074) in the normal interstitial tissue and 2.05 g/dl (0.18) in its initial lymphatics. These were from 10 random measurements per animal, using the electron microscopic densitometry method (Casley-Smith 1977b, 1979; Casley-Smith and Crocker 1975), taking the plasma in blood vessels as a standard — assumed to be 7.0 g/dl. Many cells (Figs. 2–5) were present in lymphoedema — particularly fibroblasts, macrophages, and some small and (fewer) medium lymphocytes; there were few granulocytes. As often found when excess protein lies in the tissues (Casley-Smith and Gaffney 1980), many of the macrophages contained large lipid deposits (Figs. 2, 3). There were also many cells looking like adipocytes, which probably were macrophages which had ingested much protein and converted it to fat.

Qualitative Studies

Qualitative observations showed that there were many more blood capillaries and lymphatic
Fig. 2 Lymphoedematous tissue showing two macrophages (M), one of which contains some fat, and an adipocyte (A). There are considerable amounts of protein and small collagen fibres in the tissue. 3600 x

tics, of all sizes in the lymphoedematous tissue than in the normal. The endothelium of both kinds of vessels seemed to have more vesicles and considerably more vacuoles than normal (Figs. 9, 10), and both kinds had many more endothelial open intercellular junctions (Figs. 7, 10). Some of these junctions were completely open in the plane of the section (Fig. 7); others were only open over parts of their depths, but were probably open in another plane: Fig. 10 (Casley-Smith and Window 1976; Collan and Kalima 1974). As found by Altorfer et al. (1977), the basement membranes of the initial lymphatics in the lymphoedematous tissues were often thicker than normal (Fig. 7). The walls of the larger lymphatics (Figs. 8, 9) showed much high-protein oedema, considerable fibrosis, with much distortion of the (often oedematous) smooth muscle cells (as is seen in obstructed veins — Casley-Smith et al. 1979) and some evidence of fibromuscular hyperplasia (Yamau-chi 1976). No evidence of lymphatic thrombosis was observed.

Quantitative Studies

Unfortunately, when taking the specimens the thicknesses of the sites (e.g. from the deep

Fig. 3 Lymphoedematous tissue with a macrophage which has many small lipid deposits in it. 6000 x
fascia to the surface) were not accurately measured, because the importance of this was not realised. It has been shown (Casley-Smith and Gaffney 1980) that in fact it is much more meaningful to multiply the relative volumes or numbers per cm³ by the thickness of the site (treating skin and fascia separately, and adding the products), than to follow the normal method in quantitative papers and just present the results per unit volume. The lymphoedematous tissue was much thicker (some 2 cm in most instances) than the normal (approximately 2 mm). With this huge difference in mind, it seemed to be more realistic to multiply the quantitative data for cell numbers, collagen volume, vessel lengths,
etc. by these thicknesses. Then it could be seen just how much these quantities really increased in the affected sites compared with normal. Since the concept of multiplying by the accurate thicknesses did not exist when this work was performed, we shall simply present the quantitative data in the normal manner — noting that the actual increases of each unit area of deep fascia were some ten times greater.

The quantitative data are presented in the Tables. It can be seen that they confirm the qualitative impressions mentioned earlier. In particular the relative volumes and the lengths per unit volume of the blood vessels and lymphatics were very significantly increased. The relative volumes of the collagen fibres and fat cells were raised, while that of the ground substance was reduced. This latter, which was largely oedema fluid, was actually increased superficial to each unit area of deep fascia — when the relative volume was multiplied by the thickness, but was lessened in proportion to that of the cells, fibres and vessels. Both the numbers and relative volumes per unit volume of the macrophages, fibroblasts and small lymphocytes were increased; only the increases in the numbers of the monocytes and medium lymphocytes were significant. The volumes and numbers of the fibrocytes were reduced.

The results for the vessels show that free and total numbers of small vesicles increased in both blood capillaries and lymphatics, while their diameters did not. Similarly, the numbers of the free vacuoles increased, but not their diameters. The numbers of the attached vacuoles were not estimated since their identification was difficult. In both the post-capillary venules and the initial lymphatics the proportions of open and partly open junctions increased greatly.

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Table 1 Volumes per cm$^3$ (Vv, in cm$^3$/cm$^3$) of the major tissue elements, the numbers (Nv's, x 10$^{-6}$ per cm$^3$) of the major nucleated cells observed and the lengths (Lv's, in cm$^{-6}$ per cm$^3$) of the vessels

<table>
<thead>
<tr>
<th></th>
<th>Blood vessel</th>
<th>Lymph vessel</th>
<th>Collagen</th>
<th>Ground substance</th>
<th>Fat cells</th>
<th>Macrophage</th>
<th>Monocyte</th>
<th>Fibroblast</th>
<th>Fibrocyte</th>
<th>Small lymphocyte</th>
<th>Medium lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Vv</td>
<td>0.0251</td>
<td>0.0122</td>
<td>0.0625</td>
<td>0.696</td>
<td>0.000</td>
<td>0.0045</td>
<td>0.00339</td>
<td>0.00315</td>
<td>0.00632</td>
<td>0.00129</td>
<td>0.00293</td>
</tr>
<tr>
<td>(S.E.)</td>
<td>0.00591</td>
<td>0.00417</td>
<td>0.00945</td>
<td>0.00174</td>
<td>0.0000</td>
<td>0.00254</td>
<td>0.00220</td>
<td>0.00212</td>
<td>0.00300</td>
<td>0.00136</td>
<td>0.00205</td>
</tr>
<tr>
<td>Lv and</td>
<td>0.0227</td>
<td>0.00352</td>
<td></td>
<td></td>
<td>4.21</td>
<td>3.97</td>
<td>10.2</td>
<td>46.3</td>
<td>9.01</td>
<td>7.32</td>
<td></td>
</tr>
<tr>
<td>Ny (S.E.)</td>
<td>0.0151</td>
<td>0.00593</td>
<td></td>
<td></td>
<td>0.205</td>
<td>0.199</td>
<td>0.319</td>
<td>0.680</td>
<td>0.300</td>
<td>0.271</td>
<td></td>
</tr>
<tr>
<td>Lymphoedema Vv</td>
<td>0.123***</td>
<td>0.159***</td>
<td>0.297***</td>
<td>0.119***</td>
<td>0.0779***</td>
<td>0.103***</td>
<td>0.00962</td>
<td>0.00533</td>
<td>0.0000</td>
<td>0.00987*</td>
<td>0.00783 N.S.</td>
</tr>
<tr>
<td>(S.E.)</td>
<td>0.0124</td>
<td>0.0138</td>
<td>0.0173</td>
<td>0.0122</td>
<td>0.0105</td>
<td>0.0120</td>
<td>0.00371</td>
<td>0.0110</td>
<td>0.0000</td>
<td>0.00375</td>
<td>0.00334</td>
</tr>
<tr>
<td>Lv and</td>
<td>0.121**</td>
<td>0.0549*</td>
<td></td>
<td></td>
<td>1.44</td>
<td>0.352</td>
<td>1.25</td>
<td>0.00</td>
<td>0.924</td>
<td>0.533</td>
<td></td>
</tr>
<tr>
<td>Ny (S.E.)</td>
<td>0.0347</td>
<td>0.0234</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

a) The degrees of freedom were 699 for the Vv's and 99 for the Ny's and Lv's. The results of the tests for significance are shown after each lymphoedema figure; N.S. implies 0.05<p, * implies 0.01<p<0.05, ** implies 0.001<p<0.01, *** implies p<0.001.

b) This included the oedema fluid; while the amount actually increased, because of the increase in the thickness of the site, its amount per unit volume was reduced.

c) Lv refers to the blood vessels and lymphatics only; Ny refers to the rest, except that it only applies to cells whose nuclei were visible.

d) Probably macrophages, whose nuclei were not in the section (Casley-Smith and Gaffney 1980).

Table 2 Blood vessels and lymphatics. Numbers (x 10$^{-6}$/cm$^2$ of lumenal surface) and diameters (in nm) of small vesicles and vacuoles, and the proportion of open* junctions

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Normal</th>
<th>Blood vessels</th>
<th>Lymphoedema</th>
<th>Normal</th>
<th>Lymphatics</th>
<th>Lymphoedema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free small vesicles - numbers</td>
<td>54.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>12.3</td>
<td>112***</td>
<td>67.4</td>
<td>128***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free small vesicles - diameters</td>
<td>52.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>3.87</td>
<td>14.2</td>
<td>11.7</td>
<td>14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total small vesicles - numbers</td>
<td>160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>12.3</td>
<td>305***</td>
<td>204</td>
<td>367***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free vacuoles - numbers.</td>
<td>0.0892</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>0.0723</td>
<td>2.05***</td>
<td>0.0722</td>
<td>1.84***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free vacuoles - diameters</td>
<td>186</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>13.0</td>
<td>0.321</td>
<td>0.0436</td>
<td>0.259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of endothelial junctions open and partly open</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.E.)</td>
<td>0.352***</td>
<td>0.0539</td>
<td>0.321***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations see page 137.
Discussion

The usual alterations of the tissues in chronic lymphoedema were observed as reported before (Altorfer et al. 1977; Asano 1974; Casley-Smith 1977a, 1980a; Casley-Smith et al. 1974, 1977, 1978a; Cremer et al. 1973, 1974; Földi 1969, 1975; Huth 1972, 1980; Kalima 1971; Pfleger 1964; Piller 1977; Olszewski 1977; Rusznyák et al. 1967; Veress et al. 1966). In particular, the lymphatics were dilated, with many open endothelial intercellular junctions. It has been noted (Casley-Smith et al. 1974) that it depends on the state of the surrounding tissues whether the initial lymphatic junctions are frequently opened, or all held closed, even including those which are usually open. In tissue with little or only moderate fibrosis, the vessels, and hence the endothelial intercellular junctions, are forced open by the great dilatation permitted to the vessels. In very fibrotic regions such dilatation of the vessels is not permitted and the junctions are all held closed by the increased pressure of the lymph, forcing the endothelium against surrounding, relatively unyielding, tissues.

The increase in width of the basement membranes is similar to that observed in blood vessels in many inflammatory conditions and probably reflects increased concentrations of protein. Indeed, in our lymphoedematous animals, the concentrations measured in the vessels, and in the tissues, were both very high and approximately equal — reflecting the equilibration which must have been achieved via the many open junctions and the non-functional state of the lymphatics. What happens when the vessels function under normal conditions is reflected by the much higher protein concen-
trations found in the initial lymphatics as compared within the tissues. Similar results have been obtained elsewhere using this method (Casley-Smith 1977b, 1979b, 1980b). The alterations in the walls of the larger, collecting, lymphatics are similar to those observed in walls of obstructed veins and probably are caused by increased protein concentration (Casley-Smith et al. 1979).

It is of considerable interest that the macrophage numbers are so much increased in the lymphoedematous animals, and that they contain so much lipid. Similar transformations of macrophages to cells very like adipocytes were also seen in the experiments with excessive accumulations of proteins (Casley-Smith and Gaffney 1980) and it is known that accumulations of fat (presumably from protein fragments) are common in lymphoedema (Földi 1977). This increase in macrophage numbers was also found in the experiments using repeated injections of plasma proteins (Casley-Smith and Gaffney 1980); indeed it was found that their numbers were even more increased by treating the animals with benzo-pyrones — that these drugs reduced the amount of the chronic inflammation, including the excess collagen formation. This follows from our knowledge of how these drugs act to increase macrophage numbers and proteolytic activity in many forms of high-protein oedema (reviewed: Casley-Smith 1976; Földi and Casley-Smith 1978). Thus these cells, especially when their activity is enhanced by the benzo-pyrones, act to aid the failing lymphatics in lymphoedema, or the normal but over-loaded ones in other high protein oedemas. They are a major part of the "extralymphatic mastering of plasma proteins" (Földi 1975), or tissue proteolysis (Casley-Smith 1976; Földi and Casley-Smith 1978). It is of considerable importance that this action of the macrophages appears to reduce the excess fibrosis associated with such conditions.

The alterations in the vesicle and vacuolar numbers in the lymphatic endothelium was very similar to that which occurred in the blood vascular endothelium — with the proviso that the open junctions arose from different causes. The causes of these, in the initial lymphatics, have been discussed. The alterations in the blood vessels are just those found in acute inflammation (Casley-Smith and Window 1976 reviewed: Casley-Smith 1971a, 1980a). It has been shown (Casley-Smith and Carter 1979) that the vacuoles do transport large amounts of macromolecules across the endothelium, while the open junctions are permeable in both directions and probably contribute less nett outward protein flux than was thought (Gaffney 1980).
Hitherto there have been no electron microscopical studies of chronic inflammation (except for the excellent, quantitative ones in gingiva by Schroeder’s group — Schroeder and Minzel-Pedrazzoli 1973; Schroeder et al. 1973a, b). Our results are similar to theirs to the qualitative results in chronic inflammation (Florey 1970; Spector 1969), and to those in the experiments where plasma was chronically injected into subcutaneous tissue (Casley-Smith and Gaffney 1980; Gaffney and Casley-Smith 1980). One must conclude that they are all closely linked. This is even reflected by the increase in the lengths of blood vessels and lymphatics per unit volume of tissue, just as found clinically and by casts of the blood system in experimental lymphoedema (Clodius 1980). There are minor differences in numbers, but the similar direction and degrees of alteration of all the important tissue constituents strongly indicate that chronic inflammation is indeed caused by accumulations of altered protein (as suggested by Willoughby and Di Rosa 1970), and that chronic lymphoedema is one form of this.

Acknowledgements

We are most grateful of the skilfull technikal assistance of Mrs. G.A. Piller and Mr. K.W.J. Crocker. J. Casley-Smith was supported by the Australian Research Grants Committee, L. Clodius by the Swiss National Scientific Fund and N.B. Piller by Schaper & Brümmer, West Germany

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