Tissue Changes in Chronic Experimental Lymphoedema in Dogs

J.R. Casley-Smith[1], L. Clodius[2] and N.B. Piller[3]

[1] Electron Microscope Unit University of Adelaide, South Australia
[2] Unit of Plastic Surgery (Dept. Surgery B), University Hospital Zurich, Switzerland
[3] School of Health Professions, Sturt College of Advanced Education and Flinders Medical Centre, Bedford Park, South Australia

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

Dedicated to Prof. *M. Földi* on the occasion of his sixtieth birthday.

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

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 (*Matisons* 1980) that lymphoedema causes alterations — at least to macrophage numbers — in tissues in 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.
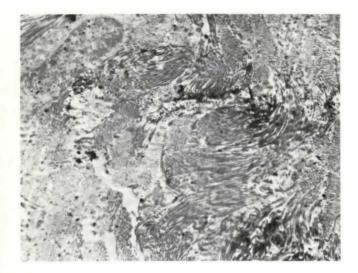
The fixative, 4 g/100 ml glutaraldehyde in Millonig's (1961) buffer, was employed for 1 hour at 4 °C. The blocks were briefly washed and were post-fixed in 2 g/100 ml OsO_4 in the buffer, dehydrated in acetone and embedded in araldite. Sections were stained with lead (pH 10) and Uranyl acetate. They 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 V_V (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 μm^2) were recorded, using the same criteria, and the N_A's estimated (numbers per unit area — *Weibel* 1969). Standard Errors were estimated using the Poissonian Distribution. The N_A values were converted to N_V'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 L_V's (lengths per unit volumes) of the vessels were estimated by doubling their N_A'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

Fig. 1 Lymphoedematous tissue showing much fibrosis. 1800 x

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 bi-modal – 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 arte-factually 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 lumenal 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 altera-tions of lymphoedema (Figs. 1–5, 7–10), with considerable fibrosis (Fig. 1). There was much oedema (Figs. 2–5), with a high protein con-centration: 5.8 g/dl (S.E. 0.34) in the lymph-oedematous 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 pres-ent in lymphoedema – particularly fibroblasts, macrophages, and some small and (fewer) me-dium lymphocytes; there were few granulo-cytes. 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.
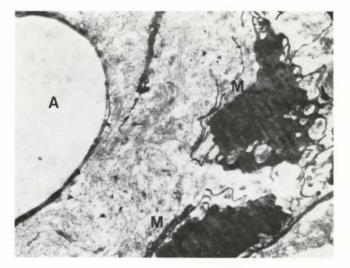
Qualitative Studies

Qualitative observations showed that there were many more blood capillaries and lympha-

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 (*Yamauchi* 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
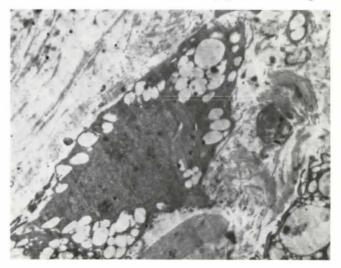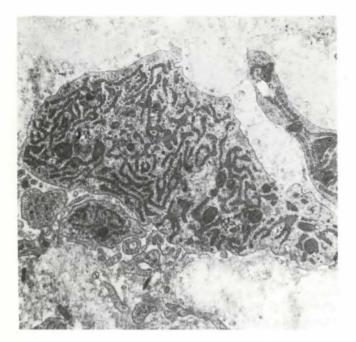

Fig. 3 Lymphoedematous tissue with a macrophage which has many small lipid deposits in it. 6000 x

Fig. 4 Lymphoedematous tissue with a fibroblast showing its typically filled endoplasmic reticulum 3000x

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,
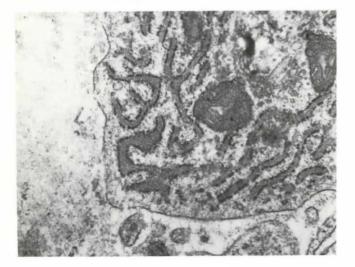

Fig. 5 A higher magnification of part of Fig. 4. 18000x

Fig. 6 Two initial lymphatics (I)
in normal tissue. 1200 x

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.
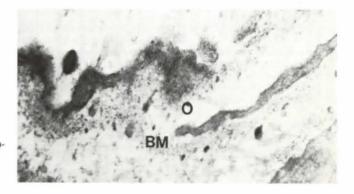

Fig. 7 Lymphoedematous tissue, with an initial lymphatic with an open endothelial intercellular junction (O) and a thickened basement membrane (BM).
18 000 x

Table 1 Volumes per cm³ (V_V, in cm³/cm³) of the major tissue elements, the numbers (N_V's, × 10⁻⁶ per cm³) of the major nucleated cells observed and the lengths (L_V's, in cm × 10⁻⁶ per cm³) of the vessels[a]

		Blood vessel	Lymph vessel	Collagen	Ground substance[b]	Fat[d] cells	Macrophage	Monocyte	Fibroblast	Fibrocyte	Small lymphocyte	Medium lymphocyte
Normal	V_V	.0251	.0122	.0625	.696	.0000	.00451	.00339	.00315	.00632	.00129	.00293
	(S.E.)	.00591	.00417	.00945	.0174	.0000	.00254	.00220	.00212	.00300	.00136	.00205
	L_V and N_V[c] (S.E.)	.0227 .0151	.00352 .00593				4.21 .205	3.97 .199	10.2 .319	46.3 .680	9.01 .300	7.32 .271
Lymphoedema	V_V	.123***	.159***	.297***	.119***	.0779***	.103***	.00962 N.S.	.0853***	.0000*	.00987*	.00783 N.S.
	(S.E.)	.0124	.0138	.0173	.0122	.0105	.0120	.00371	.0110	.0000	.00375	.00334
	L_V and N_V (S.E.)	.121** .0347	.0549* .0234				208*** 1.44	12.4*** .352	156*** 1.25	0.00*** 0.00	85.3*** .924	28.4*** .533

a) The degrees of freedom were 699 for the V_V's and 99 for the N_V's and L_V'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) L_V refers to the blood vessels and lymphatics only; N_V 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 ª (× 10⁻⁸ /cm² of lumenal surface) and diameters[b] (in nm) of small vesicles and vacuoles, and the proportion of open[c] junctions[d]

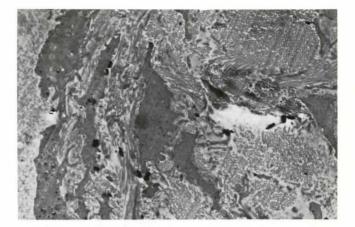|	Blood vessels		Lymphatics	
Organelle[e]	Normal	Lymphoedema	Normal	Lymphoedema
Free small vesicles - numbers	54.5	112***	67.4	128***
(S.E.)	12.3	14.2	11.7	14.8
- diameters	52.3	51.8 N.S.	52.1	53.2 N.S.
(S.E.)	3.87	2.97	2.36	3.07
Total small vesicles - numbers	160	305***	204	367***
(S.E.)	12.3	21.3	15.7	23.9
Free vacuoles - numbers	.0892	2.05***	.0722	1.84***
(S.E.)	.0723	.321	.0436	.259
- diameters	186	194 N.S.	183	215 N.S.
(S.E.)	13.0	14.9	15.3	18.3
Proportion of endothelial junctions open and partly open	.000	.352***	.0539	.321***
(S.E.)	.000	.0462	.00953	.0498

Abbreviations see page 137.

Fig. 8 Lymphoedematous tissue showing the distored wall of a collecting lymphatic, with oedema, fibrosis and some fibromuscular hyperplasia. 1800 x

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-

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Abbreviations to Table 2, page 136:
a) Allowing for the effects of "lost caps" (*Weibel* 1969) and multiplying the numbers of free vesicles by 0.82 to allow for random angles of sectioning (*Casley-Smith and Davy* 1978).
b) These are the root-mean-square, internal diameters, measuring from between inner aspects of unit-membranes, multiplying by 0.97 and adding 1.8 nm — to overcome the effects of random angles of sectioning (equation 21; *Casley-Smith and Davy* 1978).
c) A junction was considered open if any portion of it exceeded 30 nm in width, since it was considered — as shown by serial sectioning — that it was completely open in a plain other than of the section (*Casley-Smith and Window* 1976; *Collan and Kalima* 1974).
d) The degrees of freedom were 24 for the numbers, 99 for the small vesicular diameters and the junctional proportions, and 49 for the vacuolar diameters. The results of the tests of significance of the lymphoedematous against the normal conditions are shown after the latter mean, with the asterisks having the same significance as in Table 1.
e) The blood vascular results are for capillaries, except for the proportion of open junctions, which were in post-capillary venules; the lymphatic results are for initial lymphatics only.
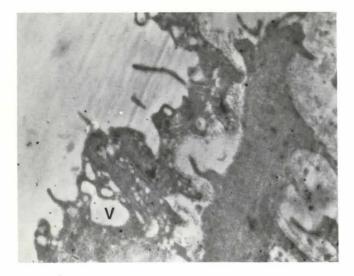
Fig. 9 The endothelium of a collecting lymphatic, as in Fig. 8 showing the excessive numbers of vesicles and vacuoles (V). 18000x

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 (*Casely-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 acrosse the endothelium, while the open junctions are permeable in both directions and probably contribute less nett outward protein flux than was thought (*Gaffney* 1980).
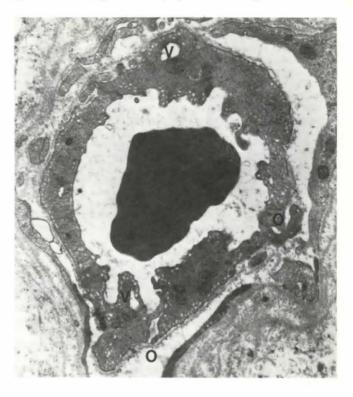
Fig. 10 A blood capillary in lymph-oedematous tissue has 2 endothelial intercellular junctions partly open (O) which are probably completely open in another plane, many vesicles and some vacuoles (V). 9000 x

Hitherto there have been no electron micro-scopical studies of chronic inflammation (ex-cept for the excellent, quantitative ones in gingiva by Schroeder's group — *Schroeder and Münzel-Pedrazzoli* 1973; *Schroeder* et al. 1973a, b). Our results are similar to theirs, to the qualitative results in chronic inflamma-tion (*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 vol-ume of tissue, just as found clinically and by casts of the blood system in experimental lymphoedema (*Clodius* 1980). There are mi-nor 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 suggest-ed by *Willoughy and Di Rosa* 1970), and that chronic lymphoedema is one form of this.

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J.R. Casley-Smith, D.Sc., M.B., Electron Microscope Unit, University of Adelaide, South Australia