FURTHER OBSERVATIONS ON LIVING LYMPHATIC VESSELS IN THE TRANSPARENT CHAMBER IN THE RABBIT'S EAR—THEIR RELATION TO THE TISSUE SPACES [1]

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TEN FIGURES

INTRODUCTION

The development of the method for inserting transparent chambers, or windows, into the ears of rabbits, as worked out in this laboratory (Sandison, '28; Clark, Kirby-Smith, Rex, and Williams, '30) has made possible the same type of precise, long-continued microscopic study of cells and tissues in the living mammal which had previously been carried out in the naturally transparent tail fins of amphibian larvae (Clark, '09, '12, etc.). Preliminary observations on the new growth of mammalian blood vessels were made by Sandison ('28), and a number of workers in this laboratory have since described the growth of new blood vessels and the subsequent changes in the vascular pattern over a period of months in sixty 'round table' chambers of the same size and shape and of known and controlled thickness (Clark et al., '31).

In the course of observations upon the growth of new blood vessels and their further changes in a chamber of the 'round table' type, undoubted lymphatic vessels, continuous with similar vessels in the surrounding preformed tissue were observed on the table area. Since the 'round table' in this

[1] The work in this laboratory on the method of studying living cells and tissues in the transparent chamber inserted in the rabbit's ear is being aided by a 5-year grant from the Rockefeller Foundation.

form of chamber occupies the space left by a hole cut clear through the ear at the time of the operation for insertion of the chamber, all of the tissues which develop in it are newly formed (regenerated) structures. Thus positive evidence for the regeneration of mammalian lymphatics was obtained. It soon became evident that the same type of long-continued cytological study of the growth of lymphatic vessels was possible in the artificial transparent chambers as had previously been carried out upon the growing lymphatic vessels in the transparent tails of tadpoles (Clark, '09, '12, '22).

In the past two years, lymphatic vessels have been identified in most of the chambers inserted, while in a dozen cases prolonged microscopic studies of individual lymphatic vessels over a period of months have been carried out. Preliminary accounts of such studies were presented in 1931 before the American Association of Anatomists and the Physiological Society of Philadelphia, and a description of the growth of lymphatic capillaries, as seen for the first time under the oil immersion lens in the living mammal, has recently been published (Clark and Clark, '32). From careful prolonged study of the same growing vessels and of the neighboring blood vessels and surrounding connective tissue with the oil immersion lens and with the aid of frequent camera lucida and photomicrographic records, it was definitely established that new lymphatic vessels invaded the observation space in the chambers by extension from preexisting lymphatic endothelium and that they grew by the sprouting of blind ending tips, accompanied by mitotic division of their endothelial nuclei, in the same manner as the lymphatic vessels of amphibian larvae, and that, throughout their growth period and later life in the transparent chambers, they formed a definite, specific system of vessels. Lymphatics were found to be less labile than blood vessels—they sent out fewer sprouts, anastomosed less frequently, and their growth was more easily interfered with, but, once formed, they showed less tendency toward retraction or toward changes in size and form than did the blood vessels.

As reported, we found that the growth pattern of the new lymphatics in different chambers varied with differences in the consistency of the connective tissue present in the invaded regions. In chambers in which the lymphatic grew onto the 'table' relatively early, when the spaces between the actively growing and rapidly changing blood vessel network were occupied by new-forming connective tissue of a loose character, the lymphatics showed a tendency to grow at random and to form a wide-meshed plexus, while in cases of late invasion, after densely packed connective tissue fibers had formed between the blood vessels, the growing lymphatics showed a tendency to advance as single vessels in a loose space along one or more of the larger blood vessels. In chambers in which the detached collars were used (Clark and Clark, '32) and the growth of connective tissue was greatly retarded and much less dense, the new lymphatics formed a relatively rich plexus, even when their ingrowth took place some time after the complete vascularization of the table area.

A description has also been given of the modification in the form of individual growing lypmhatics in adaptation to the comparative looseness or density of the surrounding connective tissue. In the loose connective tissue the contour of the lymphatic vessel was smooth, while in cases in which the outflow from the table was interfered with, wide sac-like bulgings occurred somewhat suggestive of embryonic lymph sacs. In regions of denser connective tissue the contour of the lymphatic was constricted, at intervals, by connective tissue bands. In cases in which the connective tissue in the path of the growing lymphatic vessel was particularly dense, the vessel stopped growing and acquired a bulbous end, which persisted unchanged for weeks.

It was found from the direct observations of living growing lymphatic vessels in the rabbit's ear, that they grew out as a definite independent specific system of vessels and that, although the richness of the resulting lymphatic plexus and the form of individual vessels composing it might be modified by the character of the connective tissue outside, the growth

of the lymphatics occurred, as in Amphibia, by direct sprouting from preexisting endothelium and quite independently of blood vessel endothelium or of any type of connective tissue cell.

Thus, regardless of the still unsettled question of the exact mode of origin of the first lymphatics in the embryo—whether from venous endothelium or from mesenchyme cells—these studies showed definitely that the mode of growth (or regeneration) of lymphatics in the rabbit is the same as that suggested by Ranvier ('95) and confirmed by the injection experiments of Sabin ('02, '04) in the pig embryo, Polinski ('10) in bovine embryos, and by Mierzyjewski ('09) in the chick, and demonstrated conclusively by direct observation of the living growing vessels in the transparent tail fins of amphibian larvae (Clark, '09, '12), viz., by the sprouting of endothelium 'just as the roots of a tree penetrate the ground' (Ranvier).

In addition to making possible observations on the mode of growth of mammalian lymphatics, the present method affords an unequaled field for investigation of their morphological and physiological properties. A number of preliminary observations of this kind have been carried out and still others are in progress which will be reported later. The present investigation is concerned with further observations upon the morphology of living lymphatic vessels with especial regard to the tissue outside.

Before the first discovery of lymphatic vessels (the lacteals by Aselli in 1622 and other lymphatics by Rudbeck in 1653) the name 'lymph'—for colorless fluid—was in common use, and later was retained and attributed, more or less indiscriminately, to the fluid in true lymphatic vessels, to the fluid in the cavities of the eye, ear, and joints, to cerebro-spinal fluid, to the fluid of the perineural spaces, and to the 'tissue fluid,' which was presumed to fill the spaces between cells of the subcutaneous tissue. After the discovery of definite lymphatic ducts, a hazy conception of the small terminal vessels ('lymph radicles'), which were supposed to be open at the

end and to fade out into the tissue spaces, persisted for some time, due to lack of favorable methods for demonstrating them. With the studies of Sabin ('04) and MacCallum ('03), strong evidence was presented for 'closed lymphatics'—vessels separated from the outside tissue everywhere by a definite endothelial membrane—while by the method of long-continued observation on living vessels of the transparent tails of amphibian larvae, where the finest details of the vessel walls with their endothelial nuclei can be seen with marvelous clearness, it was possible to establish, beyond a doubt, the fact that the lymphatics of this region, like the blood vessels, are all lined by a definite endothelial membrane (Clark, '09, '12).

In investigations extending over many years, in which numerous individual vessels in many different amphibian larvae were followed by prolonged daily observations under both normal and a variety of experimental conditions, only two instances of a definite opening in a lymphatic were observed. The first of these was noted upon one of the numerous occasions in which the picking up of extruded erythrocytes by a lymphatic capillary was being followed. In this case, in which one red blood cell was seen to enter the lymphatic quite rapidly, a distinct opening in the tip, which remained for half a minute, was seen (Clark, '09, p. 194). In the second case, an injury to the tip of the tail involving the most posterior lymphatic capillary had occurred which resulted in a localized area of edema. On the day following the injury, the lymphatic had a bulbous end instead of the usual pointed tip, and with the oil immersion lens it was possible to see several minute openings to the exterior through which small pigment granules from the tissue just outside were seen to enter the end of the lymphatic vessel and to move along inside its lumen. On the day following, the edema had subsided and the openings to the lymphatic were no longer visible (Clark and Clark, '27, pp. 371–372). Such openings are evidently exceptional and transient, in the case of lymphatics of the tadpole's tail.

Moreover, careful microscopic studies of living lymphatics in amphibian larvae showed that their wall was composed of two layers, i.e., a clear, homogeneous exoplasm and a basket-work-like anastomosing network of endoplasm containing the nuclei (Clark, '11). This structure was demonstrated strikingly on one occasion when a large macrophage was seen to make its way through the wall of a lymphatic and into the lumen where, as it moved along, it was observed to push an endothelial nucleus with surrounding endoplasm completely off the inner wall of the vessel without leaving any visible hole (Clark and Clark, '27, p. 370). It was found that, in the growing lymphatic of the tadpole's tail, the endothelium evidently forms a syncytium, since no silver markings of cell boundaries can be demonstrated, since the endoplasmic fibers anastomose, and since individual nuclei have been seen to move past one another in the wall of living vessels (Clark, '12).

The conception of a closed system of lymphatic vessels has been generally accepted for the past 20 years. Sabin ('16) has reviewed the modern morphological evidence for this viewpoint and has emphasized the importance of distinguishing between 'tissue fluid' outside and 'lymph' as the content of the system of lymphatic vessels. Recently, however, Drinker ('31) has revived the older conception of the interchangeability of tissue fluid and lymph. While admitting the existence of anatomically closed lymphatics, he considers that the terminal lymphatic vessels are so delicate as to be continually ruptured, and hence physiologically open, and that the content of the lymphatic vessels draining any given region of the body is a 'cross section' of the tissue fluid there present.

CHARACTER OF LYMPHATIC ENDOTHELIUM IN THE LIVING MAMMAL

Until the development of the method of introducing transparent chambers into rabbit's ears, there existed no satisfactory region for the study of the finer microscopic details of the terminal lymphatics in the living mammal. Hence it was not

known to how great an extent the observations on the characteristics of lymphatic endothelium of amphibians were applicable to those of mammals. However, in the standard 'round table' chamber, in which the thickness of the observation space, into which the new tissue and vessels grow, was regulated and maintained at 40 to 75 μ, the same lymphatic vessels could be studied, with high magnifications, both during their period of ingrowth and often for months afterward, in the living mammal.

The lymphatic vessels in such chambers normally show as clear channels lined by a complete wall of endothelium in which the endothelial nuclei, seen in profile, stand out as clear, flattened, lens-shaped structures or, occasionally, as oval swellings which bulge into the lumen. At times, when such a bulging nucleus was located at a narrow point in a vessel which contained a few leucocytes, the soft jelly-like character of the nuclear material was demonstrated, for the cells were seen to indent the nucleus as they squeezed by. Nuclei on the wall toward the observer (en face) are more difficult to see, but, when visible, they show as clear oval structures similar in their appearance to those found in amphibian vessels. Delicate protoplasmic prolongations from the region surrounding the nucleus, similar to those found in Amphibia, were sometimes visible. From careful day-by-day camera lucida records, made with the oil immersion lens, of the same lymphatic vessels, it was clear that the endothelial nuclei were not fixed structures, but that, like those of Amphibia, they changed their position in the vessel wall. Records of mitotic division of the endothelial nuclei were given in the preceding article (Clark and Clark, '32).

The outer contour of the mammalian lymphatic capillary, as seen in the chambers, is smooth—the numerous fine lateral processes characteristic of the amphibian lymphatic being absent. Irregularities in contour sometimes seen were found to be due to the presence of unyielding bands of connective tissue which formed constrictions around it, a description of the formation of which has been given (Clark and Clark,

'32, p. 279). The walls of the lymphatic vessels were found to be composed of endothelium alone, regardless of their diameter (compare figs. 1, 2, and 3). The longitudinally arranged adventitial cells (the so-called Rouget cells) found on the blood capillaries of Amphibia (Rouget, 1873; Vimtrup, '22; Clark and Clark, '25) and of the rabbit (Sandison, '31) are absent on the living lymphatics of the table area. (This is true also of the lymphatic vessels of the tadpole's tail.)

That lymphatic endothelium is slightly distensible is clear from the great differences in caliber of different vessels. Moreover, on occasion the same vessel has been observed to enlarge to 2 or 3 times its former caliber in the course of days (figs. 5 and 6), or even hours following a period of inflammation, while still retaining the same delicate wall. Such enlarged vessels have also been observed to return to a narrower caliber following the disappearance of the inflammatory condition or blockage responsible for the distention. However, this property of moderate elasticity appears to be less marked in the case of the lymphatic endothelium than in that of the blood capillaries.

The lymphatic vessels, which grew into the space over the table, anastomosed with neighboring lymphatics or ended blindly either in a somewhat tapering point, characteristic of the growing tip, or as a rounded bulb (figs. 4 and 5). Figures 1 to 6 are all oil immersion photomicrographs of typical living lymphatics, with one or more neighboring blood vessels and show the differences in caliber of different vessels and the characteristic appearance of the endothelial walls. They also show differences in cellular contents of different lymphatics. Observations have been made on the cellular content of lymphatics, the kinds of cells present, and their mode of entry, and will be reported separately in connection with studies upon the activity of mammalian lymphatics. It is of interest here, however, to note that flow in the lymphatics present in the transparent chambers is frequently sluggish and often consists of a back and forth movement with no real progression and that in many cases the same cells have

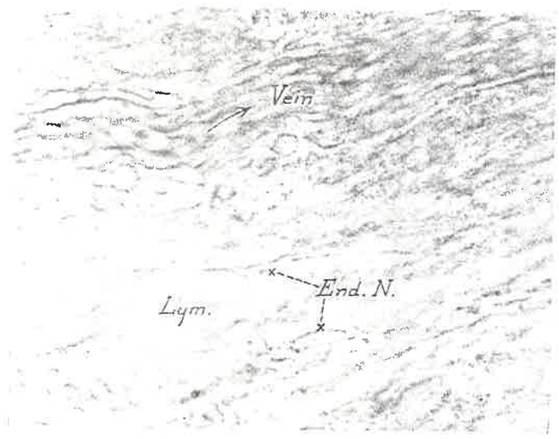

Fig. 1 Oil immersion photomicrograph of living lymphatic capillary and venule. Chamber in ear for 20 days. New lymphatics grew onto table area 6 days before this picture was taken. *Lym.*, lymphatic; *End.N.*, endothelial nuclei of lymphatic. × 720. Figures 1 to 6 were all taken with the Leitz Mifilmca camera, oil immersion lens, no. 8 ocular, $\frac{1}{25}$ second exposure.

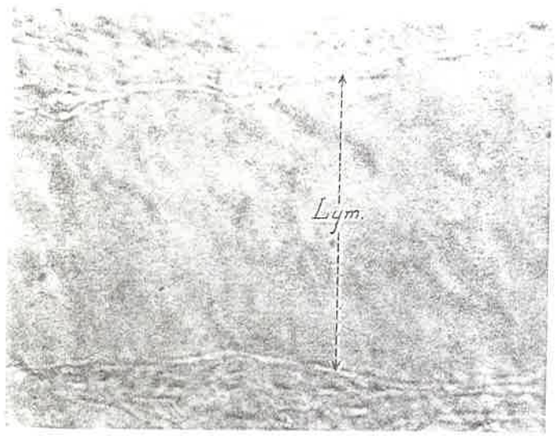

Fig. 2 Oil immersion photomicrograph of living lymphatic capillary, markedly distended. This lymphatic grew into a 'moat' chamber, installed by Mr. R. G. Abell 76 days previously. *Lym.*, lymphatic. × 720.

Fig. 3 Oil immersion photomicrograph of living lymphatic capillary. Chamber in ear 26 days. New lymphatics grew onto table area 13 days before picture was taken. *Lym.*, lymphatic; *L.End.N.*, lymphatic endothelial nucleus; *Leuc.*, leukocyte in lumen of lymphatic; *C.T.*, connective tissue (fibers differentiated). × 720.

Fig. 4 Oil immersion photomicrograph of a rounded tip of a living growing lymphatic capillary. Chamber in ear for 20 days. New lymphatics grew onto table area 6 days before picture was taken. *Lym.*, lymphatic capillary tip; *Mac.*, macrophage in lumen of lymphatic. × 720.

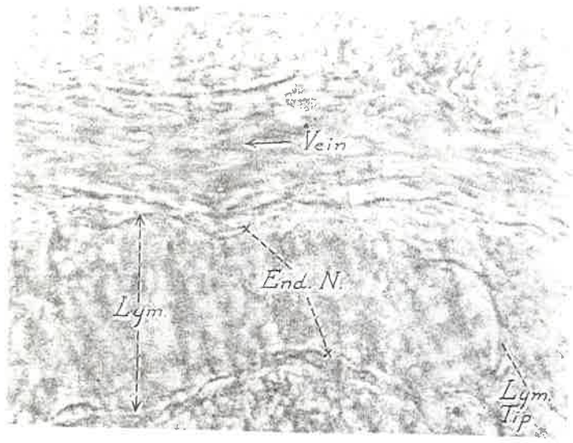

Fig. 5 Oil immersion photomicrograph of bulbous end of living lymphatic capillary, near vein. Chamber in ear for 32 days. New lymphaties grew onto table area 18 days before picture was taken. *Lym.*, lymphatic; *Lym.tip.*, lymphatic tip; *End.N.*, endothelial nucleus of lymphatic. Compare with figure 6 (same region 6 days later). × 720.

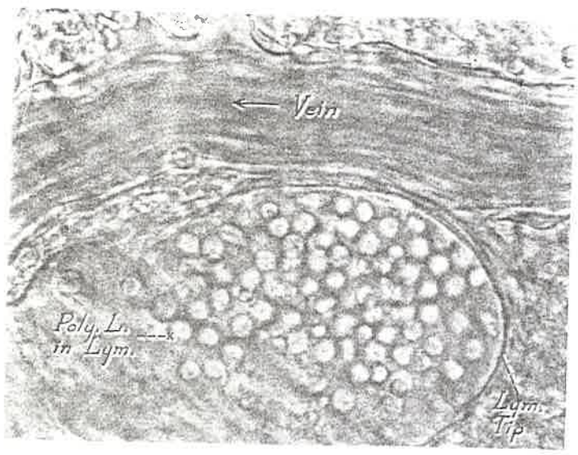

Fig. 6 Oil immersion photomicrograph of same region and vessels shown in figure 5. Picture taken 6 days later, following experiment which caused edema of the whole ear resulting in temporary blockage of lymph flow and distention of lymphaties of the observation area. The same bulbous end of the lymphatic, shown in figure 5, is greatly distended and contains cells. *Lym.tip*, lymphatic tip; *Poly.L.*, polymorphonuclear leukocytes in lumen of lymphatic. × 720.

been identified inside one of the bulbous ends, such as that shown in figure 6, for days and even weeks.

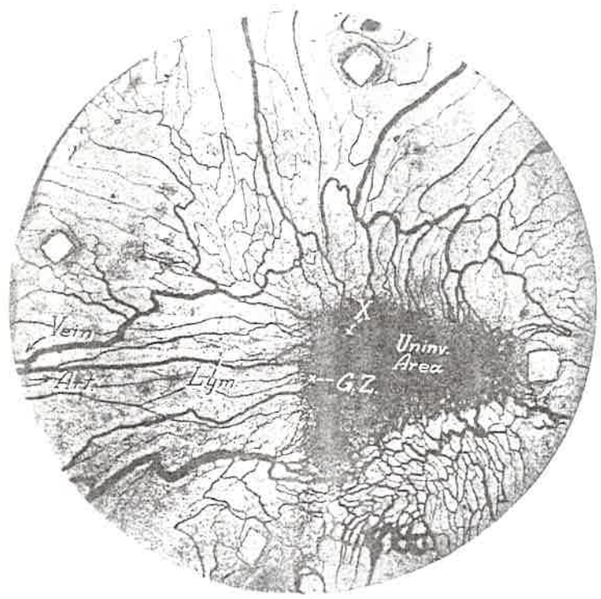

Fig. 7 Low power photomicrograph of entire table area, showing location of the growing lymphatic, open at the tip, illustrated in figure 8. Photograph taken on July 8th, 22 days after installation of the chamber. (See fig. 8, same date.) *Lym.*, lymphatic; *Art.*, artery; *Uninv.*, uninvaded area; *G.Z.*, growing zone of blood vessels; *X*, region of the uninvaded area in which movement of cells and fluid in and out of open lymphatics was observed(arrows in fig. 8). × 6.5.

ARTIFICIAL HOLES IN LYMPHATIC ENDOTHELIUM—COMPARISON WITH BLOOD VESSEL ENDOTHELIUM

In long-continued intensive observations of the same living vessels it was found that, under normal conditions, lymphatic capillaries of the rabbit's ear, like those observed in Amphibia, formed a completely closed system. The few exceptions to this rule, which will now be described, were so rare that they served to emphasize more strikingly the usual condition.

In the course of observations on growing lymphatics in the transparent chamber, one interesting exception to the usual condition of closed lymphatics was encountered. In this instance, new lymphatics invaded the table area at a relatively early stage—14 days after the operation and 6 days after the first appearance of blood capillaries on the table—and the period of their further growth coincided with that of many of the advancing blood vessels. At 18 days after the operation, the growing lymphatic vessels kept pace with the blood capillaries so that their tips reached the central area of the table, which had not yet been covered by the invading zone of new blood vessels and connective tissue and which, as described (Clark et al., '31), was still occupied by blood clot, serum, fibrin network, and varying quantities of freely movable erythrocytes and leukocytes. On this day, a definite opening in the tip of one of the advancing lymphatic vessels was noticed which communicated freely with the extravascular space over the central table area. We were able, with the oil immersion lens, to follow individual blood cells as they were forced into this open lymphatic capillary, where they moved for a considerable distance along the vessel lumen and then back again into the free fluid outside.

On the day following, the opening into this particular lymphatic vessel had closed and, although the same vessel was studied carefully every day for over two weeks, no reopening could be detected. However, another lymphatic, located near the first, was observed on this day to have an undoubted opening at its end which communicated freely with the fluid present in the region beyond the outermost zone of growing blood capillaries. Individual erythrocytes, bobbing freely in the fluid of this uninvaded area, were seen to enter the lymphatic, where they moved along for relatively long distance, occasionally even passing off the table, into the communicating lymphatic vessels of the preformed tissue. In most cases, however, after moving along the lymphatic for a distance of 2 or 3 mm., the course of these blood cells was reversed, and they moved back to the lymphatic tip and out again into the

free central space, where they spread out over a wide fan-shaped area, as shown in the camera lucida drawing (fig. 8, July 5). The 'bobbing' of cells was caused by the heart beat, while the extensive movements of cells was the result of alternating dilatation and contraction of arteries which produced a raising followed by a lowering of the mica cover. When raised, the cells moved out the peripheral ends of the lymphatics, and when lowered they were forced from the extravascular space into the lymphatics.

This second open lymphatic vessel was observed on succeeding days and daily camera lucida records of it and of the neighboring blood vessels and lymphatics were made. The opening at its tip persisted for 6 days. During this time this lymphatic vessel, still open at the tip, together with several other vessels and a whole plexus of blood capillaries, continued to encroach upon the uninvaded area, which became progressively smaller. During this time many of the free erythrocytes were phagocytized by macrophages. It was found that the endothelium of this open lymphatic grew out along the very path taken by the blood cells which had moved back and forth in the uninvaded area (fig. 8, July 8, '13). During the succeeding days, coincident with decrease in the size of the central area containing free fluid and blood cells, fewer and fewer cells moved in and out of the lymphatic. Finally, it united with a neighboring lymphatic sprout which had been growing out simultaneously and parallel with it. At the same time the free fluid outside disappeared, as shown by the stillness of cells there, and the lymphatic became closed, no new cells entering it (fig. 8, July 13th).

Although we have made intensive long-continued microscopic studies of many other growing and new-formed lymphatics, we have not seen a repetition of such an open vessel in any of our studies upon the lymphatics in the 'round table' chambers. We have, however, observed several instances of temporary artificial openings into preformed lymphatics following the operation for the installation of another type of chamber—i.e., the 'preformed tissue' chamber, in which

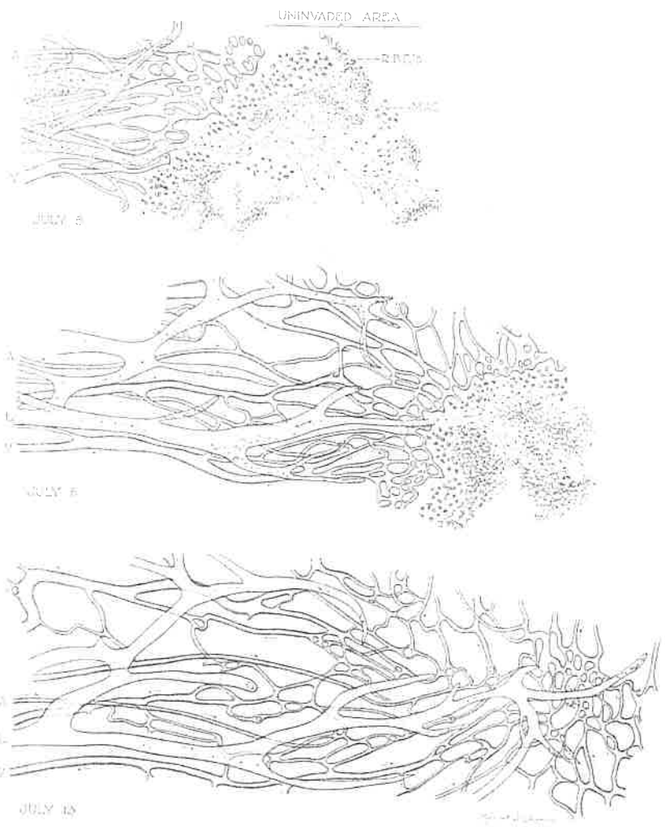

Fig. 8 Series of records made with Leitz drawing eye-piece of the same growing lymphatic capillary tip on succeeding days. This lymphatic was torn open at the tip and individual blood cells and fluid were seen to move back and forth between the lumen of the lymphatic and the uninvaded area (see text, p. 286). Compare July 8th with figure 7. *L.*, lymphatic; *A.*, arteriole; *V.*, venule; *Mac.*, macrophage; *R.B.C.*, red blood cells. Arrows indicate paths of individual blood cells forced in and out of the open tip of the *lymphatic*, by raising and lowering of mica cover, following circulatory changes. × 45.

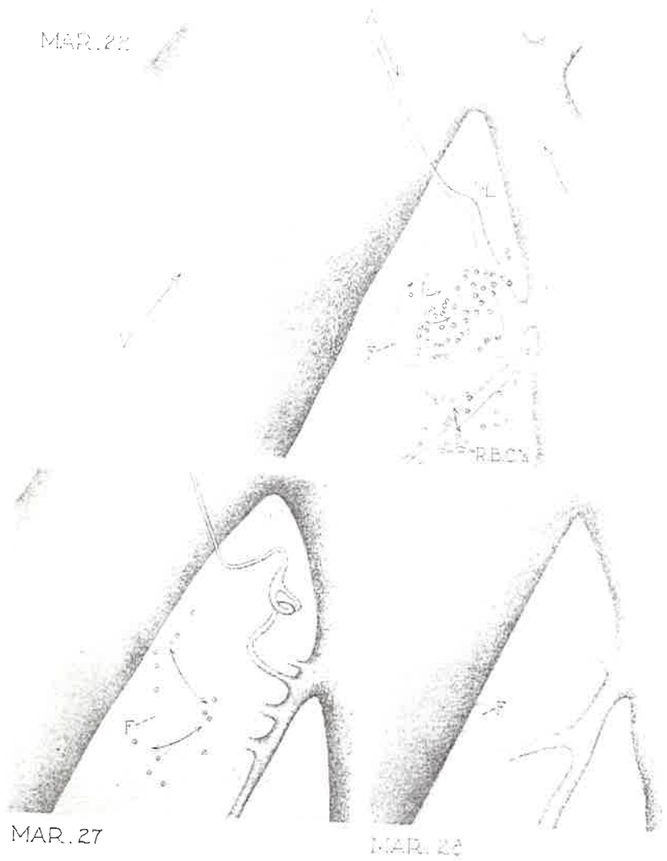

Fig. 9 Series of records made with Leitz drawing eye-piece of an artificial hole or foramen (*F*) in a preformed lymphatic (*L*) injured at the time of operation for insertion of a 'preformed tissue' chamber on March 17th. (See text, p. 287.) March 22nd. Blood cells (*R.B.C.*) bobbing with the heart beat beneath the mica cover were seen to enter and leave the lumen of the lymphatic (*L*) through the opening (*F*). Arrows indicate paths of individual blood cells. March 27th. A few blood cells still moving in the fluid layer, but none entering lymphatic. Hole (*F*) in lymphatic still shows, but new tissue has formed between vessel and fluid layer. March 28th. Hole smaller. No free cells. The following day hole could not be seen. *A*, arteriole; *V*, vein. × 65.

the original tissue of the ear, including blood vessels, nerves, and lymphatics, is retained for observation within a thin space (Clark et al., '30, p. 200). In the operation for the installation of this variety of chamber, the skin and cartilage are removed from the inner side of the ear, while the outer skin with its vessels, nerves, and surrounding tissue is retained virtually intact. With improved technique, greater experience, and the use of the 'binocular loupe' during the operation, injuries to the tissue have been reduced to a minimum. However, unavoidable localized injuries to minute vessels and other tissues do occur. Moreover, vessels—both blood and lymphatic—pass through the cartilage, and these are inevitably broken during the operation. The results of such injuries to blood vessels and their subsequent recovery have been described (Clark and Clark, '32).

In several instances in chambers of this variety, we noted, on the first few days following the operation, the presence of a definite opening into a preformed lymphatic vessel. The exposed surface is kept flooded with sterile Ringer's solution during the operation, and for several days after the installation of the chamber free fluid is present over the tissue and directly beneath the mica cover, the presence of which is clearly discernible by means of the extravasated blood cells which are moved about in it by changes in the blood flow. In the case of such an artificial hole made into a preformed lymphatic at the time of operation, the movement back and forth of individual blood cells from this layer of free fluid into the lumen of the injured lymphatic and back out again, could be clearly seen. Such artificial openings were observed to persist for days (2 to 4 in the cases studied) during which time their size gradually diminished. Figure 9 shows a record of such an artificial tear of a preformed lymphatic vessel which occurred at the time of operation and persisted for 9 days. At 10 days the region of the hole was still visible, but appeared to be covered by a thin layer of tissue as the free particles had ceased to pass back and forth between the lymphatic lumen and the free fluid over the surface. In

a recent experiment of this type, a lymphatic was located just beneath the cartilage removed at the operation. Five days later, a group of fourteen openings was present, through which cells passed back and forth between the vessel lumen and the fluid outside. The holes lay so close together as to give to the vessel the appearance of a sieve (fig. 10). A day

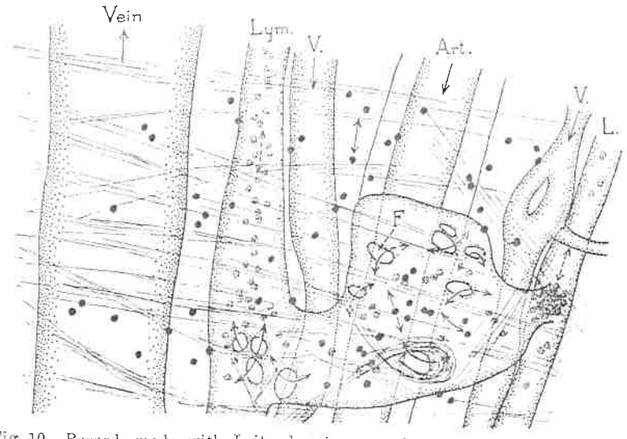

Fig. 10 Record, made with Leitz drawing eye-piece, of a lymphatic injured at the time of operation for insertion of chamber, showing a number of openings between the vessel lumen and the fluid space outside through which blood cells were seen to move back and forth. Chamber inserted April 11th, drawing made April 16th. The following day the holes had closed. *L.*, smaller branch lymphatic; *Lym.*, lymphatic; *V.*, venule; *Art.*, arteriole; *F.*, foramina, or artificial holes, in sac-like enlargement of lymphatic. Arrows indicate back and forth movement of blood cells between lumen of lymphatic and layer of free fluid over surface of tissue. Solid dots indicate extravasated blood cells present over surface, the stippled ones those inside the lymphatic. × 130.

later, although the outlines of the holes still showed, a thin layer of tissue had appeared, separating the vessel from the free fluid still present just beneath the mica cover and no more cells moved in and out. The following day, the holes had closed. Later than the first week or 10 days following this type of operation, no such opening in the lymphatics of the preformed tissue have been seen.

In the case of the openings into lymphatics following the operation for installation of the 'preformed tissue' chambers, it was clear that they were definite artificial tears. That this was also true for the exceptional case of the open tips of growing lymphatic capillaries (fig. 8) just described seems probable, since they were noted in a chamber in which there was considerable movement, caused by pulling, on the table area. In all of the modified 'round table' chambers in which stresses were removed by the use of the detached protective collars (Clark and Clark, '32) the resulting growth area was much quieter and in none of these has any indication of a persistent opening into a lymphatic been observed, although free fluid was present outside in a number of cases.

That the persistence of an opening into a lymphatic is dependent upon the presence of free fluid outside was shown not only by the closure, in the case mentioned, when the free fluid disappeared, but by the behavior of lymphatics broken open when they were surrounded by the normal tissues, cells, and intercellular substance.

In chambers in which the growth of blood vessels and the growth and differentiation of connective tissue has been completed, and in which there has been no inflammatory condition, the lymphatic capillaries are surrounded by connective tissue fibers, various types of wandering cells, etc., but no free fluid. Frequently there are erythrocytes in the bulbous ends of the lymphatics. Many times, following a sudden pressure on the mica cover, we have seen erythrocytes forced out of the lymphatic into the tissue space. Always under the circumstances described—that is, with the normal, non-fluid extravascular material—the lymphatic tip closed at once, and the extruded erythrocytes remained outside, either directly against the lymphatic or nearby, until they were phagocytized by macrophages or went to pieces.

These observations have shown that, although mammalian lymphatics are normally closed, they may be torn open or forced open by outside pressure and that they close immediately if outside conditions are normal, but that if free fluid

is present outside at the time, such an artificial hole into a lymphatic may remain open to the extravascular space for days. In this respect, lymphatic endothelium differs from blood vascular endothelium, for artificially produced holes in blood capillaries are closed immediately, whether the capillaries are invading an area occupied by densely packed connective tissue fibers, or undifferentiated connective tissue cells (a condition resembling that present in the tadpole's tail) or a region filled merely by serum, fibrin, and extravasated blood. Rapid closure is also characteristic of the preformed blood vessels squeezed or torn during the operation for insertion of the 'preformed' tissue chamber— in which case the injured vessels are in contact with free fluid for several days.

Not only does the lymphatic endothelium close quickly when forced open by artificial pressure, under normal extra-lymphatic conditions, but the temporary holes produced by the migration of macrophages through the vessel wall are apparently closed at once. This latter process has been studied much more exhaustively in the case of the lymphatics and blood vessels of Amphibia and the details described (Clark and Clark, '28, '30).

CHARACTER OF THE CONNECTIVE TISSUE SUBSTANCE

As previously stated, the rare instances in which artificial holes in lymphatic endothelium failed to close immediately were noted only in cases of injured lymphatics, subjected to massage, and in which free fluid was present immediately outside the lymphatic. Cytological studies of living subcutaneous tissue both in the transparent tails of amphibian larvae and in the chambers introduced into rabbit's ears have yielded convincing evidence that free fluid is not a normal constituent of such tissue. Hence, the old terms of 'tissue fluid' and tissue 'lymph' appear to be misnomers—at least under usual conditions.

Szily ('08) and others have pointed out the presence of a clear homogeneous semi-gelatinous ground substance or 'matrix' which precedes the original formation of connective

tissue fibers in the embryo. Baitsell ('21, '25) has made extensive studies of this primitive tissue substance in amphibian and chick embryos and has demonstrated it by microdissection in the living. The presence of such a soft, jelly-like substance before the formation of typical connective tissue is described in most of the recently published text-books of histology, and for the most part the authors state that there is a continuous transition in the physical consistency of intercellular supporting substance from the primitive soft gelatinous ground substance, represented by 'Wharton's jelly' of the umbilical cord, through areolar and dense fibrous tissue to cartilage and finally bone. However, in the case of areolar connective tissue alone—the typical 'subcutaneous tissue'—the statements are usually made that the spaces between cells are 'bathed in lymph' or that the cells and tissues are 'nourished by tissue fluid.' This conception of the presence of fluid everywhere in the spaces of the tissue appears to be universally accepted in physiological investigations of lymph flow, permeability of capillaries, etc.

In studies on the transparent tail fins of amphibian larvae continued over a period of more than 20 years, involving prolonged intensive microscopic observation of living blood vessels, lymphatics, connective tissue cells, and wandering cells of different types, we have become convinced that the presence of free fluid in the tissue spaces of this region is an exceptional phenomenon. According to published descriptions (Clark, '12), the supporting tissue of the tail fin of amphibian larvae is of a simple type consisting of stellate connective tissue cells, distributed in a fairly even pattern, each of which contains a central portion with the nucleus, from which extend branched processes which taper into minute fibrillae which interlace with those from neighboring cells. Careful daily records of all the individual connective tissue cells of a selected area demonstrated that they were not fixed, but showed a continuous slow change in shape, accompanied by sending out of certain branches and withdrawal of others, resulting, in the course of days, in an actual

shifting of position (Clark, '12, p. 366). In earlier studies (Clark, '16) it was taken for granted that the clear intercellular spaces of this tissue contained free fluid, according to the orthodox notion. However, evidence gradually accumulated which definitely precluded this idea.

First, microinjections showed the general character of the tissue of the tail fin to be gelatinous and not fluid. On numerous occasions minute quantities of different substances of a colloidal, fluid, or semifluid nature, such as India ink, cream, etc., have been injected into the tissue of the tail, and in many such experiments camera lucida tracings before and after showed that the substance had been injected directly into the tissue spaces without the destruction of a single cell. When the small glass cannula (measuring about 20 mm. at the tip) was inserted and an attempt made to inject India ink, for example, by blowing, at first no fluid could be forced out of the needle. If the needle was then withdrawn slightly, without removing it from the tail and blowing resumed, the injection fluid then filled a small cavity the exact size and shape of the cannula. After removal of the glass needle the epidermis soon closed over the hole, and by this means it was possible to localize small amounts of foreign substance and to study the reaction of the vessels and tissue cells toward them (Clark and Clark, '18, '20, '22). In cases where excessive pressure was used, the injection fluid could be forced beyond the small cavity made by the needle and it then had a 'fuzzy' appearance, apparently caused by the formation of side pockets from the primary injection hole. The contrast when the injected material was introduced directly into a vessel (blood or lymphatic capillary) was most striking, for in this latter case, the substance (India ink, paraffin oil, cream, etc.) did not remain localized, but spread instantly along the vessel lumen. Then, too, the somewhat subjective evidence of the feel of the subcutaneous tissue, at the time of injection, compared with that of the fluid content of the vessel lumen, pointed to a difference in consistency of the two. It was also noted in microinjections in very young larvae (at

the time of hatching or earlier) that the tissue of the tail fins was much softer and flabbier than that present a week or more later.

And, secondly, the non-fluid nature of the tissue spaces, in the normal tail fin of Amphibia, was evident from direct observation with the oil immersion lens. Although the space between individual tissue cells is transparent, it does not, except in unusual circumstances, show brownian movement. On the other hand, dancing of small particles can always be seen in the lumen of stagnant vessels, in the vacuoles of many cells, in the compartments of the peculiar canalicular cells found in many amphibian larvae (Clark and Clark, '18, p. 233), and in the cytoplasm of dying cells (Clark and Clark, '30, p. 115). Movement of small granules and oscillation of extravasated blood cells in the tissue spaces were seen in the edema characteristic of some of the stages of inflammation and in localized regions after injections of various foreign substances. Such localized areas in which the characteristic movement of a liquid was present contrasted plainly with the surrounding tissue. The condition was evidently transitory, since the movement disappeared in the course of hours or days.

In the various types of transparent chamber designed for the study of new growth of tissue in the rabbit's ear (the Sandison chamber, the 'bay' chamber, the 'round table' chamber and the 'combination' chamber), a small piece of the ear tissue, varying in size and shape with the type of preparation used, was removed at the time of operation and an enclosed observation space in contact with the ear tissue was an integral part of each of these chambers. This space was filled with sterile Ringer's solution at the time of installation of the chamber and microscopic observation immediately afterward showed the presence in the space of fibrin in varying amounts and of freely movable red blood cells and leukocytes.

In the 'round table' chambers the enclosed space was of uniform shape and size and of known and controlled thickness.

From daily observations of the 'table' area it was obvious that free fluid was present at some places between the fibrin strands during the days immediately following the operation. Rounded blood cells and specks of debris oscillated with the heart beat, and migration of wandering cells took place only on the table surface and on the under surface of the mica cover. At other places, no movement was seen, indicating already a probable semi-gelatinous condition.

The regular advance of new blood vessels into and across the thin space over the 'table' from the periphery where they made their appearance on an average of 7 days after the insertion of the chamber has been followed, with frequent photographic records, in over sixty of the standard 'round table' chambers (Clark et al., '31). This invasion by newly growing blood vessels was accompanied by an influx of fibroblasts and wandering cells. The zone of new vessels and other cells advanced steadily, converging toward the center of the round table until finally the vessels from the opposite sides met and anastomosed and the last remnant of the blood clot was disposed of in part through the phagocytic action of macrophages and in part through cytolysis (Clark et al., '31, figs. 3 to 11). As the zone of growing capillaries advanced toward the center of the table, the older portions of the vascular plexus near the periphery were remodeled, through retraction of many of the vessels and the enlargement of others into arterioles and venules, while the intervascular spaces gradually became more and more filled with 'adult' connective tissue with long fibers and reticular threads, criss-crossing in every direction. Connective tissue fibers appeared first at the periphery of the table and gradually extended toward the center. The exact mode of formation of the fibers has not yet been determined. The time of appearance of this typical 'areolar tissue' and the density of it differed greatly with the amount of motion present in the individual chamber, being delayed and less dense in 'splinted' chambers in comparison with those in which continual massage was present, due to pressure and tension on the collars, which was transmitted to the chamber proper.

Careful microscopic observation showed that with the ingrowth of new vessels and connective tissue cells from the periphery into the space over the table, the free intercellular fluid, where present, progressively diminished and finally disappeared. In several of the 'splinted' chambers—in which connective tissue growth was retarded—new blood vessel sprouts were seen to grow directly out into the spaces between the fibrin strands, but soon after the invasion of the central area by vessels and sometimes even before, a change in the intervascular material outside evidently occurred, for oscillation and the bobbing back and forth of extravasated blood cells ceased entirely.

After the complete vascularization of the table area, no free fluid was present, as a rule, in the tissue between the vessels, although frequently a thin layer of fluid immediately beneath the mica cover and superficial to the vascular plexus persisted for some time. Eventually, new tissue completely filled the whole space over the 'table' and movement could be detected nowhere in the tissue outside the vessels. The change from fluid to non-fluid consistency of the intervascular material, accompanying the ingrowth of new vessels and cells, occurred in cases in which migrating fibroblasts and other wandering cells were scattered with relatively wide spaces between them (a state comparable to the normal tissue of the tadpole's tail) as well as in those chambers in which a relatively rapid and abundant formation of connective tissue fibers took place.

When stable, completely vascularized chambers were examined with the oil immersion lens, it was apparent that free fluid was present in the interior of vessels, but not, under ordinary circumstances, in the tissue outside. The fluid contents of circulating vessels was of course obvious, but it was also easy to detect the presence of free fluid in stagnant vessels by means of the vibration and the oscillation, with the respiratory movements and the heart beat, of any blood cells or smaller particles present in the interior of either blood vessel or lymphatic.

Descriptions have been published of lymphatic vessels which were isolated experimentally in the tadpole's tail and lymphatics which became cut off accidentally in the rabbit's ear chambers and of their subsequent union, in both cases, with other lymphatics which grew out toward them (Clark, '22; Clark and Clark, '32). The fluid content of such a cut-off vessel, surrounded by its complete endothelial membrane, contrasted sharply with the non-fluid character of the gelatinous or fibrous material outside. In comparing daily camera lucida tracings of such a cut-off vessel in the tadpole's tail, it was noted that it was wider on one day, returned to its former caliber, and widened again a few days later, thereby indicating the passage of fluid in both directions through its wall.

In one chamber in the rabbit's ear a transitory change from the typical fluid content of two cut-off lymphatics was observed. The vessels in question both contained a number of blood cells on the day they became isolated. Ten days later, it was noted that these blood cells were adherent and moved slowly back and forth inside the lymphatic as a single violet-colored mass. Three days later, the entire content of both vessels became motionless and opaque. Four days later, the contents of the two lymphatics were again freely movable. In the interval, one vessel had been reincorporated in the lymphatic system by uniting distally with a new sprout which had grown out to it, but the other one was still disconnected.

In the typical stable chamber after the stage of invasion was complete (3 weeks to a month after operation in the case of the standard 'round table' chambers), free fluid was seen to be present in the interior of blood vessels and lymphatics (whether circulating or stagnant) and apparently to be absent in the 'tissue spaces,' whether the latter were relatively wide or were merely minute slits between adjacent connective tissue fibers.

Although free fluid was absent from the tissue of a 'stable' chamber, its reappearance was noted, following a prolonged period of increased circulation. Inflammation, caused by overheating, for example, evidently accompanied by increased

transudation through the walls of the blood vessels, was followed by the appearance of vibration and oscillation of extravascular blood cells and by the dancing of fine specks of debris over the surface of the tissue, and occasionally in the interstices of the vascular network. On occasion, also, slight localized injuries to one or two small blood vessels were induced by pressure, which were followed by temporary stickiness of the endothelium for a short stretch, by the subsequent emigration of a few leukocytes, and by the extrusion of a few erythrocytes. In some of these cases, a transitory appearance of free fluid in the tissue just outside such an injured blood vessel was indicated by the oscillation of extruded blood cells in this localized area. The appearance of free fluid (exudate) under such circumstances is a repetition, on a more minute scale, of the condition present over the entire table area following the original operation for installation of the chamber.

DISCUSSION

Our observations seem to agree with those of Baitsell ('16, '25) as to the appearance of a 'gelatinous matrix' in wound healing—comparable to the embryonic 'ground substance'—which precedes the formation of 'adult' connective tissue. In regard to the exact manner of formation of connective tissue fibers in the transparent chambers, however—whether through the agency of fibroblasts or, as Baitsell has concluded, directly from this matrix or from fibrin, without the intervention of cells—our studies are still too incomplete to give a definite answer.

Aside from the observed change from fluid to non-fluid consistency of the intercellular substance accompanying the growth of new vessels into the space enclosed, nothing definite is known in regard to the character of this substance, in the transparent chambers in the rabbit's ear. Our observations appear to show that the fluid exudate in a wound becomes transformed into a 'gel,' which is the normal state of the contents of tissue spaces, and that in case of an increased

transudation, such as that found in inflammation, this change is reversible. The greater relative amount of this intercellular substance together with its softer consistency in young embryos and in certain stages of 'wound healing' may be one factor in the more rapid rate of cell migration observed in such cases (Clark and Clark, '20, pp. 225, 240, and '30, p. 124). Although only preliminary observations have been made upon the character of the intercellular substance of connective tissue and the changes which it undergoes, it is apparent that the present method offers a great opportunity for such studies.

The present observations have shown clearly that it is under circumstances such as those present in inflammation—of which the aseptic wound made in the insertion of the chamber into the ear is an example—in which free fluid accumulates in the tissue spaces, and that this was the condition present in the cases described here in which artificial openings between an injured lymphatic vessel and the tissue outside were found. Although such artificial holes in the lymphatic endothelium are evidently exceptional, the observation that they can occur, in case free fluid is present outside, and that the presence of such free fluid, although also exceptional, occurs in inflammatory conditions is of obvious clinical importance. The fact that delicate blood-vascular endothelium when injured may allow leakage of vessel contents, but that it always closes immediately, while the lymphatic endothelium when torn open may remain open for days, gives a histological explanation for the manner in which living bacteria, present in a localized area of infection, may enter the system by way of the lymphatic channels. While, again, the fact that such an opening was not observed in the 'splinted' chambers, gives objective microscopic justification for the clinical practice of immobilization in cases of infection.

The interesting experiments of Drinker and Field ('31) on the absorption of protein from the subcutaneous tissue have yielded evidence of differences in the permeability of the blood capillaries of different regions of the body, since they

showed that foreign serum injected subcutaneously entered the lymph channels and not the blood vessels, while it was absorbed from the peritoneum by both sets of vessels. In prolonged observations on the two types of vessels in the living transparent tails of Amphibia under both normal and a variety of experimental conditions, differences in their reactive powers were observed directly (Clark, '09; Clark and Clark, '17, '26), while many observations on differences in the properties of the two sets of vessels in the mammal are accumulating from our microscopic studies of the living vessels as seen in the transparent chambers. The difference in the manner of growth of the two sets of vessels, manifesting itself in the greater lability of the blood vascular system, has been emphasized (p. 274). Further differences in the two sets of vessels will be reported later, together with interesting differences noted between the properties of the mammalian lymphatics and those of Amphibia. In observing the same living growing capillaries day after day in both the tadpole's tail and the rabbit's ear chambers, the manner in which a lymphatic sprout always anastomoses with another lymphatic and a blood capillary with a blood capillary, and a growing lymphatic never connects with a blood capillary, no matter how great the proximity of the two, demonstrates in a striking fashion the fact that there must be an essential difference in the constitution of the two types of endothelium.

As for the hypothesis proposed by Drinker that lymph from the subcutaneous lymphatic vessels is identical with tissue fluid, it is clear from the direct observation of the living vessels reported here, that on certain occasions, in localized regions, such as a wound, infection, or burn, in which there is an increased circulation through the blood vessels and increased passage of fluid through the vessel walls followed by an accumulation of free fluid in the outside tissue, there may be injured lymphatic capillaries in such a region in which this is actually true. We have seen minute artificial tears in lymphatic capillaries of such an injured or inflamed area which remained open for days and in which there was not

only a free passage back and forth of fluid between the vessel lumen and the exterior, but also of formed elements, such as erythrocytes and fat globules. However, it is equally clear that, regardless of what may be true of the permeability of the lymphatic capillary to the various chemical elements present in the tissue outside, the assumption that 'lymph is tissue fluid' cannot be generally true in a physical sense, for not only do the lymphatics normally form a closed system, separated from the tissue everywhere by an intact endothelial membrane, but under normal circumstances all our evidence appears to indicate that there is no free fluid present in the tissue spaces.

SUMMARY

By the method of installing transparent chambers in the rabbit's ear, it has been possible to make prolonged microscopic studies of living mammalian lymphatic capillaries—to watch the same vessels day after day by a method similar to that used for previous studies in the transparent tails of amphibian larvae. The growth by sprouting of the new lymphatics which invade the thin space enclosed in the 'round table' chamber has been described recently, together with variations in their pattern associated with differences in the character of the connective tissue growth.

Lymphatic capillaries of the mammal, as seen in the living animal, appear as channels containing a clear fluid which may have a varying number of blood cells suspended in it. They vary greatly in caliber, but are all lined by an endothelial membrane, which is distensible and which appears to possess a slight degree of elasticity. The lymphatics either form anastomoses with neighboring lymphatic vessels or end blindly in a point, or rounded bulb.

In contrast to the obviously liquid character of the lymphatic contents (whether moving or stagnant) no free fluid is present in the tissue outside under usual conditions. This is true also of the intercellular substance of the tadpole's tail which is normally gelatinous.

Under certain conditions, such as that present immediately after a surgical operation and in inflammations, following overheating for example, accumulation of free fluid outside the vessels was observed. Under such circumstances, there was occasionally seen an artificial hole into a lymphatic, which remained open for from 1 to 9 days, during which time there was a free passage of fluid and blood cells back and forth between the outside tissue space and the lumen of the injured vessel. Such holes in lymphatics were probably produced either by direct tearing or by excessive outside pressure. They were not seen in the 'splinted' chambers, in which the observation area was immobilized and protected from outside trauma, nor in any chamber after the disappearance of free fluid outside the lymphatics. Persistent holes in the blood vascular endothelium were never seen, although injuries to blood vessels were more common than to the lymphatics, owing to the greater number present in the region of the experiment. These microscopic observations emphasize the clinical importance of immobilization in the treatment of localized injuries and infections as a means of preventing the entrance of living bacteria into the system by way of a possible hole into a lymphatic vessel.

The results of the direct microscopic observation of the lymphatic capillaries and of the connective tissue, as viewed through a transparent double-walled window introduced in the living mammal, show the presence normally of intact membranes lining both blood and lymphatic vessels and separating their fluid contents from the tissue outside, and the absence, under normal conditions, of free fluid in the tissue spaces.

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