USE OF ^{99m}Tc-SULFUR COLLOID TO ASSESS LYMPHATIC DYSFUNCTION IN FILARIAL INFECTION

VERNON K. JENKINS, ADAM EWERT*, RALEIGH F. JOHNSON, JR.** and DEAN S. FOLSE***

Department of Radiation Therapy, *Department of Microbiology, **Department of Radiology, ***Department of Pathology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77550, U.S.A.

INTRODUCTION

Filarial nematodes which develop and reside in lymphatic vessels are a major cause of lymphatic dysfunction in tropical regions of the world. Alterations of lymphatics that result from filarial infections in experimental animals have been studied by conventional lymphography (Gooneratne *et al.*, 1971; Schacher *et al.*, 1973; Koehler, 1968; Ewert *et al.*, 1972), but the technique does not permit direct measurement of the drainage efficiency of the regional lymphatic system. Redington *et al.*, (1975) introduced a technique in which ^{99m}Tc-sulfur colloid was used to assess lymphatic function in Patas monkeys infected with *Brugia malayi* infection and shows that both the route and the rate of lymph flow were altered by filarial infection and correlated with anatomical changes.

MATERIALS AND METHODS

Animals and filarial infection

Young adult Patas monkeys, both male and female, that weighed 3 to 4 kilograms were used in this study. A total of 15 hind limbs in eight animals were included in the study. One monkey remained as an uninfected control and seven monkeys were infected on one or both hind limbs with *Brugia malayi* by placing 50 infective larvae in a drop of saline onto the webbing of the feet which had been punctured to simulate mosquito bites. Some of the limbs were reinfected several months after an initial infection. The method of obtaining infective larvae has been previously described in detail (Ewert and El Bahari, 1971; Ewert *et al.*, 1972). Briefly, *Aedes aegypti* mosquitoes were fed on an experimentally infected cat and after the parasites had developed to the infective stage, the mosquitoes were dissected in saline to liberate the larvae. After infection the monkeys were individually caged and venous blood samples were examined periodically for the presence of microfilariae to confirm a positive infection.

The investigation adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal resources, National Academy of Sciences, National Research Council, U.S.A.

Assessment of lymphatic drainage

At time periods ranging from one to 21 months after an infection, the efficiency of the lymphatic drainage system in the hind limbs was assessed by injecting 50-100 μCi of ^{99m}Tc-sulfur colloid (Tesuloid, Squibb) into the webbing between the toes of the hind feet. The progression of isotope from the injection site, through the lymphatic vessels and into the regions of the draining popliteal and abdomino-pelvic lymph nodes was followed for three hours. Gamma emissions from the ^{99m}Tc-sulfur colloid was detected by a gamma camera. Appearance of the isotope in the different areas was recorded on film at 10-

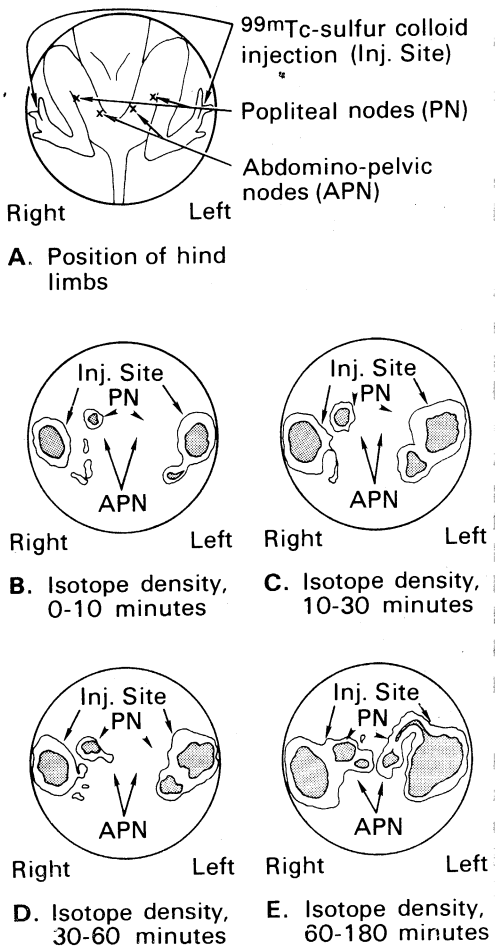

Fig. 1—A. Illustration of position of the hind limbs under the collimator during detection of isotope migration.

B-E. Density of gamma emissions at different time periods up to 3 hours after isotope injection. Darkened areas indicate greatest concentration of radioactivity. The right limb was uninfected (control) and the left limb was infected with *Brugia malayi* 7 months previously.

minute intervals and the accumulated counts were recorded on a computer at 2-minute intervals.

Some limbs were repeat tested after a single infection, some were again tested before and at various time periods after reinfection. During testing, the animals were anesthetized with sodium pentobarbitol and placed on a V-grooved board for immobilization during the 3-hour period of monitoring. To accommodate the restraints of a 13-inch in diameter collimator for the gamma camera, the hind limbs of the animals were usually folded to encompass the injection sites (hind feet), the first central lymph nodes (popliteals) and the abdomino-pelvic lymph nodes within the field of the collimator (Fig. 1A). Some of the limbs were also tested in a straight position, in which the collimator encompassed only the popliteal and the abdomino-pelvic areas to determine whether folding the limbs impeded flow through the vessels.

Anatomical evaluation of lymphatic changes

Within a few days after the final isotope studies were completed for a given animal, the animal was euthanized and the lymphatics were examined. About 10 minutes prior to an overdose of sodium pentobarbitol, lymph-staining dye (sky blue^{R}) was injected into the webbing between the toes of the hind limbs in order to identify the lymphatic vessels and identify nodes and their drainage pattern. The skin of the hind limbs and the pelvic region was reflected to reveal the lymphatic vessels draining into the popliteal and superficial inguinal lymph nodes. The peritoneal cavity was also opened to determine the pattern of drainage into other abdomino-pelvic nodes.

Lymphatic vessels and nodes were examined *in situ* under a dissecting microscope. The presence of living or dead worms and their location, abnormal lymphatic draining patterns, obvious lymphatic obstructions, tortuosity, and formation of collateral vessels were noted and photographed. After *in situ* examination, representative portions of the vessels and nodes were excised and fixed for light and electron microscopic studies. Re-

sults of the microscopic studies will be reported elsewhere.

RESULTS

Limbs that were infected for 1-9 months usually had more radioactive colloid within the vessels draining the site of isotope injection and had different patterns of migration of the colloid into the lymph nodes than did the control limbs. A representative example in which the left limb was infected for seven months and the right limb was uninfected (control) is shown in Fig. 1B-E. During accumulation of the labeled colloid in the regions of the popliteal and abdomino-pelvic nodes of the control limb, only traces of the colloid were detected in the draining vessels throughout the test period. In contrast, within 60 minutes after labeled colloid injection, colloid accumulation in the infected limb was restricted to the heel region, and there was no colloid in the lymph nodes. Between 60 and 180 minutes, the labeled colloid progressed to the region of the abdomino-pelvic nodes and remained principally in the lymphatic vessels and the abdomino-pelvic region. In this example, no radio-label was observed at the site of the popliteal node.

In all nine uninfected (control) limbs that were tested in the folded position and in all six control limbs tested in the straight position, the labeled colloid appeared initially in the region of the popliteal lymph nodes, usually within 10 minutes after injection (Table 1). In six of the nine folded limbs and in five of the six straight limbs at least a trace of the isotope appeared secondarily in the abdomino-pelvic region, usually 30-120 minutes after colloid injection, but in four of the 15 control limbs the colloid did not reach the abdomino-pelvic region within the 3-hour period.

In contrast, in all eight limbs that were tested 1-9 months after filarial infection, the labeled colloid appeared initially in the abdomino-pelvic region, usually between 30 and 120 minutes after colloid injection (Table 1). In four of the eight infected limbs, the colloid appeared in the region of the popliteal nodes about 10-40 minutes after the initial appearance in the abdomino-pelvic region, however, the colloid never appeared in the popliteal region of the other four limbs.

In seven of eight limbs that were tested 11-21 months after infection with the parasites, the labeled colloid appeared initially in the area of the popliteal nodes and secondarily in the abdomino-pelvic region, a pattern similar to untreated limbs but at a slower rate of appearance (Table 1).

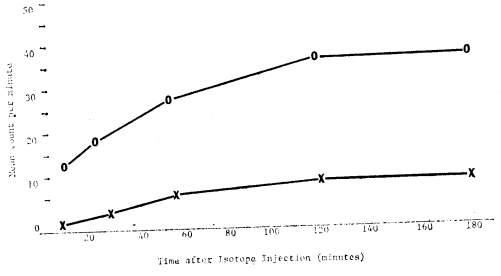

Fig. 2—Mean numbers of counts per minute in relation to time after isotope injection in the popliteal (o) and abdomino-pelvic (X) nodes of nine uninfected hind limbs of Patas monkeys.

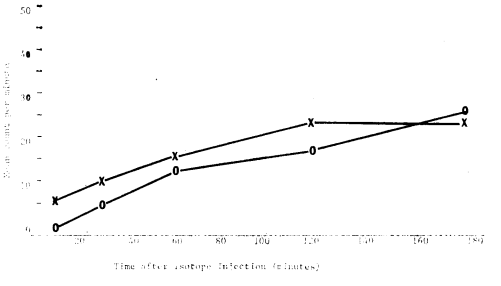

Fig. 3—Mean numbers of counts per minute in relation to time after isotope injection in the popliteal (o) and abdomino-pelvic (X) nodes of eight hind limbs infected with filaria.

Table 1

Appearance of ^{99m}Tc-Sulfur colloid in lymph nodes of Patas monkeys[a].

Time After Injection of ^{99m}Tc-Sulfur Colloid	Untreated Controls				Infected, 1-9 Month[e]		Infected, 11-21 months[f]	
	Folded limb[c]		Straight limb[d]					
	popliteal node	abdomino-pelvic node	popliteal node	abdomino-pelvic node	popliteal node	abdomino-pelvic node	popliteal node	abdomino-pelvic node
< 10 min	+−++ (8/9)	− (0/9)	+−++ (3/6)	− (0/6)	− (0/8)	+ (1/8)	+ (2/8)	− (0/8)
20− 30 min	+−+++ (9/9)	+ (1/9)	+−+++ (6/6)	+−++ (2/6)	+− (1/8)	++−+++ (4/8)	+−++ (6/8)	+ (2/8)
30− 60 min	+−++++ (9/9)	+ (3/9)	+−++++ (6/6)	+−+++ (3/6)	+−++++ (3/6)	+−++++ (6/8)	+−+++ (7/8)	+−++ (3/8)
60–120 min	++−++++ (9/9)	+−++ (5/9)	+−++++ (6/6)	++−+++ (3/6)	+−++++ (3/8)	+−++++ (8/8)	+−+++ (7/8)	+−+++ (4/8)
120–180 min	++−++++ (9/9)	+−++ (6/9)	+−++++ (6/6)	+−+++ (5/6)	+−++++ (4/8)	+−++++ (8/8)	+−++++ (8/8)	+−+++ (5/8)

Degree of Positive radioactivity[b]
(No. Animal Positive/No. Animals in Group)

[a] 50-100μCi of ^{99m}Tc was injected into the webbing of the hind foot andradioactivity in the region of the lymph nodes was detected by a gamma camera.
[b] Radioactivity: + − 10 CPM; ++ − 30 CPM; +++ − 50 CPM; ++++ − 70 CPM.
[c] Fivelimbs were tested once and two limbs were tested twice for a total of nine limbs tested. all of which were tested in the folded position.
[d] Four limbs were tested once and two limbs were tested twice for a total of six limbs tested in the straight position.
[e] Seven of the eight limbs were tested with hind limbs folded and one was tested with the hind limbs straight.
[f] All eight limbs were folded during the test.

The mean values for rate of accumulation of the labeled colloid in the region of the lymph nodes of the folded control limbs is shown in Fig. 2 and the values observed in limbs infected 1-9 months before testing are shown in Fig. 3. A consistent pattern of rapid appearance and accumulation of the colloid in the popliteal nodes and sparce, if any, appearance in the abdomino-pelvic nodes for control limbs is contrasted by an initial and greater accumulation of the colloid in the abdomino-pelvic nodes and sparce, if any, appearance in the popliteal nodes of the infected limbs.

Alteration of lymphatic structures in infected limbs was verified at necropsy. The anatomical findings for the animal shown in Fig. 1 is demonstrated in Fig. 4. In the control (right) limb, the blue dye progressed directly through two very fine, lightly stained vessels into the popliteal lymph node which stained dark blue. The lymphatic vessels were not traceable between the popliteal and inguinal nodes but the inguinal node had a trace of light blue stain. The deep abdominal nodes remained unstained. This example was typical of the dye-staining pattern of the uninfected limbs except that none of the others showed any detectable stain in the inguinal nodes. Blue dye in the lymph vessels of the infected (left) limb revealed four major vessels, two of which were dilated and tortuous and darkly stained in the region of the tubercalus and lower leg. The lymphatic vessels of the infected limb also had contained pockets of stained lymph measuring 3-4 mm in length, and five worms were located between the heel and the popliteal node which remained unstained. None of the infected limbs had worms beyond the popliteal nodes. The infected limb had dye progression through the lymphatic vessels to a region near the popliteal node, but the vessels bypassed the node. The vessels were not visible in the thigh region, but a relatively large vessel reappeared from within the deep tissue about one cm below the inguinal node and about one-third of the total mass of the inguinal node(s) was stained dark blue. This example typifies the structure of the lymphatics of limbs tested 1-9 months after infection, i.e., increased number and volume of vessels in the region of the tubercalus, unstained or lightly stained popliteal nodes and darkly stained inguinal lymph nodes. Limbs that were infected for 11 or more months had lymphatic structures and dye staining patterns similar to the uninfected control limbs.

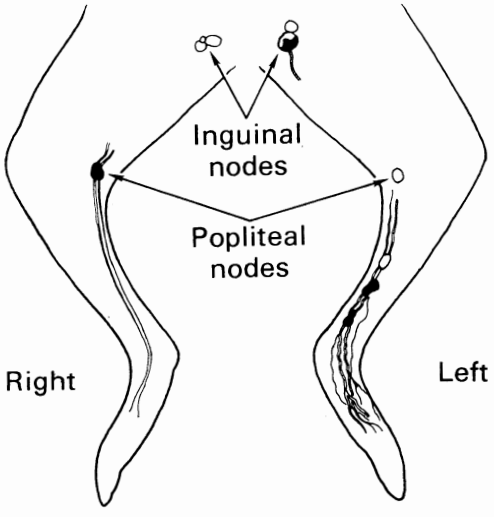

Fig. 4—Illustration of lymphatics observed in the hind limbs of a Patas monkey at autopsy. the uninfected limb (right) had a large concentration of blue dye in the popliteal node, but only a trace of dye in the inguinal nodes. The vessels of the infected (left) limb were engorged with blue dye and the inguinal nodes were stained, but the popliteal node was unstained.

DISCUSSION

One difficulty in the utilization of animal models to study lymphatic filariasis and human filarial diseases is the failure of infected animals to develop persistent lymphedema and elephantiasis. Lymphedema has been

observed in cats and dogs but the condition was always transitory (Schacher *et al.*, 1968; Rogers and Denham, 1974). The Patas monkeys used in the present study were found to be susceptible to infection with the human filarial nematode *Brugia malayi*, but overt lymphedema did not accompany the infection. The major question of this study was whether a commonly used tracer technique utilizing ^{99m}Tc-sulfur colloid could be used to assess subtle lymphatic alterations caused by sub-clinical infection and thus avoid the sacrifice of animals and the laborious task of dissecting lymphatic vessels and nodes to quantitate the effects of the infection. Such a method would be extremely useful in studying the effectiveness of anthelminthic regimens in individual animals or patients treated for lymphatic filariasis.

The preliminary report of Redington *et al.*, (1975) showed that *Brugia malayi* infection of Patas monkeys effected a reduced efficiency of lymph drainage in the infected limb. The reduced efficiency occurred in the absence of lymphedema. Our data confirm the preliminary data of Redington *et al.*, (1975) in that the rate of progression of the labeled colloid from the injection site to the regions of the popliteal and abdomino-pelvic nodes was reduced in infected limbs, especially for limbs that were infected for less than nine months (Figs. 1 & 3). Further, the pattern of migration in the infected limbs was different from the pattern in the control limbs. In the control limbs there was a consistent and rapid initial appearance of the labeled colloid in the region of the popliteal node and a secondary appearance of the isotope in the region of the abdomino-pelvic nodes in most of the limbs (Table 1, Fig. 2). In addition there were only traces of the colloid detected in the lymphatic vessels. In contrast, limbs tested 1-9 months after infection with filaria consistently had greater accumulation of the labeled colloid in the draining vessels and an initial appearance of the label in the region of the abdomino-pelvic nodes (Table 1, Fig. 3). In the limbs infected for 1 to 9 months the isotope appeared subsequently in the popilteal region in four of the eight limbs but did not appear in the popliteal region of the remaining four limbs (Table 1). Limbs that were infected for 11-21 months usually had a pattern of appearance of labeled colloid similar to the control limbs; an initial appearance in the popliteal region and secondary appearance in the abdomino-pelvic region, but the rate of appearance was reduced (Table 1).

We concluded that the isotope technique was indeed useful in detecting alterations of the lymphatic system induced by filarial infection. Our data showed that the normal, unimpeded pattern of lymph drainage from the feet was directly through patent lymphatic vessels to the popliteal nodes which trapped most of the colloid and only after engorgement of the popliteal node did the colloid migrate to the second level of nodal drainage. For limbs infected 1-9 months the colloid migration rate and pattern suggested that the normally patent lymphatic vessels that drain directly into the popliteal nodes were at least partially occluded by parasites, or reaction to the parasites, and that collateral vessels channeled the colloid more slowly, but directly to the second level of nodal drainage (abdomino-pelvic region). It appeared that in the four limbs that eventually accumulated colloid in the popliteal region, the primary vessels were still functional, but in the four limbs that did not accumulate colloid, the vessels were occluded to the extent that under these conditions there was no lymph flow. The near-normal pattern of migration in limbs infected for 11-21 months suggested that recovery from the infection was taking place.

Dissection of the limbs after sacrifice confirmed the normal pattern of flow observed by

the tracer technique in control limbs (Fig. 4). The blue dye was carried directly to the popliteal nodes staining them dark blue and secondarily less dye appeared in the inguinal nodes. Also, as indicated by the tracer technique, limbs that were infected for 1-9 months had altered lymphatic structures. The dye was prevalent in numerous lymphatic vessels between the infection site and popliteal nodes and the popliteal nodes remained unstained or were lightly stained with blue dye. Several worms were consistently found lodged in the dilated vessels between the injection site and the popliteal node. Since the dye-stained vessels did not enter the popliteal nodes, the indication from the tracer technique that newly formed collateral vessels, or pre-existing vessels that are normally inactive, channeled the dye directly to the inguinal node without filtering through the popliteal node was supported.

These data and the data of Redington *et al.*, (1975) indicated that the ^{99m}Tc-sulfur colloid technique is useful and accurate for assessing lymphatic dysfunction even in the absence of lymphedema. The technique permits assessment of rate and pattern of lymph flow in the intact animal and since animals can be repeatedly tested, has the advantage of application to studies of anthelminthic therapy.

SUMMARY

Investigations of lymphatic dysfunction in animals infected with filarial parasites has been hampered by a paucity of techniques to measure efficiency of lymphatic drainage. In this study a ^{99m}Tc-sulfur colloid technique was used to assess the efficiency of lymphatic drainage in Patas monkeys infected with filarial nematodes. In all 15 uninfected hind limbs there was rapid and consistent appearance of labeled colloid in the primary lymph node (popliteal) and subsequently in the secondary nodes (abdomino-pelvic) in 11 of 15 limbs. In contrast, in all eight limbs tested 1-9 months after infection there was reduced rate of migration of the colloid and initial appearance in the abdomino-pelvic region: subsequent accumulation was seen in the popliteal region in only four of the limbs. This data indicated that lymphatic vessels were blocked and that collateral vessels channeled the colloid to the secondary lymph nodes. The lymph flow patterns demonstrated by the isotope technique were supported at autopsy.

ACKNOWLEDGMENTS

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