A Comparison between Granulomatosis and Lymphoreticular Neoplasia in *Diemictylus viridescens* and *Xenopus laevis*¹

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**SUMMARY**

Stocks of *Xenopus laevis* which have been used recently to generate lymphoreticular neoplasia (L-1) have apparently become infected with pathogenic acid-fast bacteria. These materials provided us with an unusual opportunity to compare a granulomatous response with this lymphoreticular neoplasm of the histiocytic type in both newt and *Xenopus* hosts. The granuloma foci were found to differ from those of the neoplasm with regard to their cellular composition (50% histiocytes, 50% polymorphonucleocytes), growth characteristics (enlargement by compression or distortion of neighboring normal tissues), and necrosis (caseating). In both host species, there appeared to be a subsequent increase in mitosis within granuloma nodules leading to an increase in histiocyte numbers, suggesting a transition to the histopathology of L-1. Acid-fast bacterial populations appeared to be brought under control eventually by phagocytosis as their numbers decreased with time. While previous studies showed no effect of thymectomy on L-1 development in *Xenopus*, granuloma formation increased considerably as compared with nonthymectomized controls. The use of 30,000 × g centrifugation of an L-1 tissue homogenate allowed for injection of a supernatant fluid which subsequently initiated neoplastic foci and demonstrated that granuloma development need not precede lymphoid neoplasia in the newt.

**INTRODUCTION**

The incidence and the biology of lymphoid tumors in amphibia has recently been reviewed (9). Questions relating to the etiology of the various disease states reported in different species were considered, and the need for further elucidation in this area was stressed. The importance of additional etiological and histopathological studies has been emphasized by Dawe (11, 12) who surveyed the occurrence of lymphoid responses and pointed out that at least some of the diseases considered to be neoplasms appeared to be transmissible by gram-positive, acid-fast bacteria and that workers in the field have not attempted to make a distinction between infectious granulomas and lymphoid neoplastic disorders in amphibia. This distinction is, of course, crucial if one is to use the amphibian disease state as a model system to study neoplasia in general.

This report aims to extend our knowledge concerning one amphibian disease in terms of making the distinction noted above, namely of comparing it to a granulomatous response. This lymphoid disease (L-1) was the first reported for an amphibian and was originally diagnosed as a reticulum cell sarcoma or a lymphoblastic lymphosarcoma (2). The disease proved to be transmissible not only to *Xenopus laevis*, the South African clawed toad, the species in which it was first studied (5), but also to alien species of the order Anura (6) and even to Urodela, e.g., *Triturus cristatus*, the European crested newt (3, 4) and *Diemictylus (Triturus) viridescens*, the common American newt (18) (Figs. 1 and 2). Studies concerned with the biological characteristics of the transmissible agent strongly suggested that it was likely to be a small virus with an essential lipid component in the coat (7, 8). Acid-fast bacteria which have been seen as frequent but inconstant residents of the tumor have been considered to be there by virtue of secondary infection. A more detailed classification of this disease as a reticulum cell sarcoma of the histiocytic type has also been made (16).

In previous detailed studies on the development of this lymphoreticular neoplasm in *Xenopus* (15), granulomas did not appear when allo- or xenogeneic tumor tissues or homogenates had been used. While the newt proved to be...
susceptible to L-1, no detailed developmental study has been done with this species.

This report was made possible for the fortuitous initiation of granuloma from some frozen-stored *Xenopus* L-1 tissue implanted into both newt and *Xenopus* hosts. While thymectomy had little effect on subsequent L-1 development in either the newt (18) or *Xenopus* (8), it was used to test whether it had any effect upon the development of granuloma in *Xenopus*. A new developmental study of L-1 initiation in newts after injection of a *Xenopus* tumor homogenerate is also included in order to help clarify the distinction to be made between the two disease states.

**MATERIALS AND METHODS**

The *D. viridescens* specimens used in these studies were obtained from a dealer in Donelson, Tenn., and the *X. laevis* were bred in this laboratory from parents supplied by a dealer in Baltimore, Md. The frozen-stored tumor material had been generated in young, postmetamorphic *X. laevis*. It had been frozen and stored at −20°C for several months; the exact time is unfortunately not known. The thymectomies, implantation of tumor fragments, and the preparation of a 30,000 X g supernatant fluid of tumor tissue homogenerate were all accomplished by previously described techniques (8, 18). The implants were kept as nearly equal in size as possible, and in the injection experiment 0.1 ml was dispensed i.p. All of the animals were kept at 25°C in either standing tap water or distilled water, and they were fed pieces of beef liver at regular intervals. The animals were given injections of 2 μg/g body weight of colchicine (i.p.) 12 hr before they were killed, so that mitotic patterns could be observed. While Bouin’s fluid was used to kill and fix all tissues, Carnoy’s fluid or formol-alcohol was used for all internal tissues so that some slides could be stained for acid-fast bacteria, using carbol-fuchsin with hematoxylin as a counterstain. All other slides were made with standard hematoxylin and eosin stains, and all paraffin sections were 8 μ thick.

Twenty-seven newts received implants, in the left forelimb, of tumor tissue fragments. Two hosts were sacrificed every 4 days, and their implanted forelimbs, livers, spleens, and kidneys were prepared for histological examination. The study was completed at 45 days postimplantation. The 25 newts that received i.p. injections of supernatant fluid from the tumor tissue homogenate were sacrificed one at a time every other day for a 50-day experimental period. The 50 *Xenopus* hosts that received tumor tissue fragments in the left forelimb were sacrificed at weekly intervals. Twenty-five of these *Xenopus* hosts had been thymectomized 7 days prior to implantation.

**RESULTS**

By 4 days, no cellular outlines of the frozen-killed *Xenopus* L-1 tissue could be clearly discerned in the implanted newt limbs. However, host cells had begun to aggregate in the implant site, particularly polymorphonucleocytes. Through 12 days, the cellular population suggested that an inflammatory condition was present. At 16 days, a shift to nodular granulomatosis was observed as the number of histiocytes increased and a massing of the cells took place. The granulomas in the limb persisted, increasing in mass, and even though Bouin’s fluid had been used, a few intracellular acid-fast bacteria were still stainable. Some mitoses were observed in the masses within the limb. The cellular composition of the granulomas was very nearly 50% histiocytes and 50% polymorphonucleocytes (Fig. 3). The cytoplasmic volumes were large and prominently eosinophilic in the granulomas. Caseating necrosis could be seen within the mass in the limb of a 20-day host. With few exceptions, after 20 days the implanted limbs were extensively ulcerated.

Within the newt viscera, while a few mitotic figures were present in hematopoietic cells of the liver subcapsular epithelium, which in the newt and *Xenopus* is a granulopoietic site, many more mitoses could be seen in the spleen as early as 4 days postimplantation. This splenic activity remained very high throughout 16 days. The spleen is an erythropoietic and lymphopoietic organ in both species under study. Granulomatous nodules appeared in both liver and spleen by 16 days postimplantation. The picture at this point becomes somewhat complicated by the fact that one 20-day specimen possessed nodules typical of the lymphoreticular neoplasm, in that the foci were composed of an essentially pure population of histiocytes, frequently with a halo of mature lymphocytes surrounding the histiocytic mass. The other 20-day animal had developed granuloma nodules made up of about equal proportions of polymorphonucleocytes and histiocytes, with strongly eosinophilic cytoplasm. The cellular composition and the subsequent growth and caseating necrotic characteristics observed in more advanced centers suggest that all were more typical of tuberculous granulomatosis than of lymphoreticular cancer. Some of the difficulties of distinguishing between lymphoid responses and lymphoid disorders in man have recently been reviewed (10), as have nonneoplastic hyperplasias in lymph nodes of animals (20). In the newt, with the exception of the one 20-day host, foci had the qualities of granulomas through 28 days. Occasional mitotic figures could be seen associated with them, most typically on the periphery of a focus. As foci enlarged, they appeared to compress neighboring normal tissues so that distortion of liver tubules was apparent and some flattened cells surrounded them (Fig. 4). Acid-fast bacteria which were predominantly intracellular were abundant within the foci and typically were packed into both histiocytes and polymorphonucleocytes. Macrophages in sinusoids in which no foci were present were frequently bright red with packaged acid-fast bacteria, a condition more typical of “diffuse” than “nodular” tuberculosis (19). No acid-fast bacteria were found in those 20-day host foci which were typical of the L-1 disease. The bacterial population in granuloma nodules appeared to be heaviest in the 24-day animals and to diminish thereafter, with one exception, an animal that died at 31 days postimplantation. This would be the expectation following extensive caseating in mycobacteriosis. Of the two specimens sacrificed at 32 days postimplantation, one had 3 granuloma foci which exhibited some caseation and giant multinucleate...
macrophages (Fig. 5). Some caseating necrosis was observed later within the viscera of one of the 38-day animals. The other 32-day and 38-day specimens, as well as the 45-day hosts, all exhibited an apparent transition from granuloma to the characteristic lymphoreticular histopathology. In these animals, the bacterial population, although still considerable, was reduced; the mitotic rate within foci had increased; and the proportion of histiocytes to polymophonucleocytes increased dramatically (Figs. 6 and 7).

No granuloma was found in any of the kidneys. The first instances of nodular formation in a kidney were noted at 31 days; they were composed of mitotically active histiocytes with an areola of small lymphocytes.

In the newts receiving i.p. injections of tumor homogenate supernatant fluid, there was an almost immediate mitotic response in both the liver subcapsular epithelium and in the spleen. Minor accumulations of small lymphocytes and polymophonucleocytes were found in the liver subcapsular epithelium as well as in the sinusoids of the liver up to 15 days postinjection. A return to normalcy gradually took place until, at Day 26, there was a renewal of mitotic activity in the liver and several small L-1 nodules were observed in the sinusoids. A subsequent burst of mitotic activity and L-1 formation began in the liver at 49 days. No acid-fast bacteria were seen in the viscera. Splenic mitotic activity also declined at 14 days, the proportion of small lymphocytes to stem cells remaining high.

Nonthymectomized, implanted Xenopus exhibited much the same pattern already described within the implanted newt limb but with some interesting differences occurring in the viscera. The only major differences to be noted in the implant site appeared in the animals sacrificed between 25 and 42 days, where a transition to L-1 had apparently taken place. By that time, the masses in the limb were histiocytic and did not involve a substantial number of polymophonucleocytes. Invasion and metastasis within the limb was typical of that usually seen with L-1.

In the viscera, small foci appeared as early as 7 days within the liver sinusoids, and unlike the situation in the newt, where a particular animal had visceral nodules typical of granuloma or the cancer, the Xenopus developed mixtures of foci, such that some were histiocytic and others were essentially equal populations of polymophonucleocytes and histiocytes (Fig. 8). Development of both types of foci was relatively slow through 21 days. As in the newt, the granuloma foci appear to enlarge distorting adjacent normal liver tissues (Fig. 9), while more typical L-1 foci infiltrate neighboring tissue (Fig. 10). By 28 days, the mitotic rate was found to increase in granuloma nodules, providing the suggestion of the same type of transitional development noted previously for the newt viscera (Fig. 11). By 42 days postimplantation, only typical L-1 foci were produced in that organ. No caseating necrosis or giant cells were seen in the Xenopus granulomas, although one of us (L. N. R.) has previously reported (16) that they both have been seen in chronic granulomas not related to the disease state presently under study. The only instance of necrosis was typical of the beginning central noncaseating necrosis previously described for L-1 (15), which was found in one large focus at 28 days postimplantation. Almost all of the visceral foci and the limb masses as well of this animal were L-1 in their histopathology.

Thymectomy changed the Xenopus response pattern considerably. Granulomas grew much more rapidly and pervasively in the limb and viscera than they did in the nont hymectomized animals. A macroscopic comparison of the number of liver foci, apparent by 21 days is shown in Fig. 12. Here, one cannot distinguish granuloma from neoplastic centers by gross observations. In the limbs, apparent neoplasia was not observable until 35 days postimplantation. In the viscera, one 14-day animal showed liver damage which was apparently unrelated to the formation of foci of either type. Fewer animals possessed mixtures of granuloma and L-1 foci. One each of the 21- and 28-day specimens showed this type of mixed pathology. It was not until 35 days postimplantation that the mitotic rate in granulomas rose, leading to the appearance of a transitional stage. Nevertheless, by 42 days only L-1 histopathology seemed to be present in the Xenopus hosts. No necrosis was observed in the sacrificed Xenopus, although one animal that died at 35 days showed generalized noncaseating breakdown. No giant macrophages were found in these thymectomized Xenopus. While some acid-fast bacteria were seen throughout these Xenopus studies associated with both types of foci, at no time did their numbers approach the relatively enormous populations seen in the newt studies. This may be due to the fact that the bacteria were of Xenopus host origin and some partial immunity may be shared by Xenopus to the ubiquitous, varied mycobacterial strains.

**DISCUSSION**

The frozen-stored Xenopus tumor tissue used in these studies initiated growths which differed in several ways from previously described lymphoreticular cancer of the newt and Xenopus. Instead of being histiocytic centers of growth, the foci were composed of cellular populations about equally divided between histiocytes and polymophonucleocytes. Enlargement of these growing centers caused compression and distortion of neighboring epithelium in the liver; where necrosis was found within these masses in the newt, it was caseating and in at least 3 instances in a newt host there was involvement of giant multinucleate macrophages. The use of colchicine allowed a distinction to be made between the relatively small number of mitoses within these foci and the greater number typical of the neoplasm. Heavy infestations of acid-fast bacteria were also found, not only in foci but also in phagocytes scattered throughout the normal tissue, a situation not found previously in studies with the neoplasm alone. Because of these characteristics, then, one of the two diseases that developed from implants of this material is considered to be an infectious granuloma. Descriptions of diseases of bacterial etiology in amphibia are available (14).

While a transition from granuloma to the neoplastic histopathology was suggested in the study with newt hosts, the animals died or were sacrificed too early for us to observe the completion of the process. On the other hand, the study with Xenopus allowed a more thorough analysis of this
aspect, and the animals sacrificed in the late stages of that experiment exhibited masses which appeared to be neoplastic. While this might be viewed as a shift from a tuberculoid to lepromatoid mycobacteriosis, utilizing a higher proportion of histiocytes with a lower level of hypersensitivity (19), the diminishing population of acid-fast bacteria suggests that it is more likely to be a matter of bringing the bacteria under control, a situation which might allow the histiocytic populations the opportunity to respond to the L-1 agent. In drawing a distinction between granuloma and lymphoid neoplasia in these amphibians, there are two other differences which relate to developmental behavior. First, in the thymectomized *Xenopus*, the development of granuloma was clearly more rapid and more extensive than in the nonthymectomized animals. Prior studies had suggested that thymectomy had little effect on the development of L-1 foci in newt and *Xenopus* following implantation of fragments of tumor tissue (8, 18). Thymectomy in conjunction with X-irradiation is known to enhance development of L-1 foci in newt and *Xenopus* following injection of homogenates of L-1 tissue (15). Regardless of the method of transmission, the L-1 disease undergoes a period of growth and spread before a generalized noncaseating necrosis sets in which destroys foci regardless of their size or location. As is the case with tuberculoid mycobacteriosis, no scar formation takes place. Neoplastic foci then reappear and grow. The timing of the cycle of growth, necrosis, and regrowth is affected by heredity, temperature, and agent titer, but is entirely predictable for a given set of conditions. The necrosis appears to require an immunologically responsive environment (17). One aspect made clear by the results of the present studies with *Xenopus* is that this type of necrosis was not observable in any tumor centers until very late in the experimental period, well over 2 weeks later than one would have expected to find it, had the initial foci been L-1 rather than the granuloma. Concomitant granuloma seems to retard the appearance and developmental rate of the neoplasm. It would be interesting to learn how the bacteria may affect the response to the L-1 agent and to substantiate the transition suggested by the data reported here.

The results of these studies suggest, then, that we now have pathogenic bacteria in addition to the previously analyzed L-1 agent. Initially, the response to these bacteria seems capable of dominating the observable activities in the hosts, but eventually most of the animals appear to be capable of bringing the disease under control by clearing many of the bacteria from the system by phagocytosis, perhaps allowing a transition to neoplasia to take place. Tests of stock L-1 tissues currently stored in our —70°C freezer show that acid-fast bacteria are recoverable from many samples but not from others. There is no question that this contamination of our material with the pathogenic bacteria will complicate future experiments on the neoplasm until we can ensure its elimination from the material used for transmission of the lymphoid disorder. This applies particularly to the use of implants of tumor fragments.

The developmental picture in newt reported here following injection of the supernatant fluid from *Xenopus* L-1 tissue suggests that the early mitotic response in spleen and in hematopoietic cells of the liver subcapsular epithelium tended to diminish until the tumor foci arose between 4 to 7 weeks postinjection. What is especially pertinent to this report is that the development of granuloma is not a prerequisite to L-1 formation in this species. This conclusion could not have been assumed on the basis of previously reported experiments (18), since those schedules of sacrificing animals had not included the early pretumor stages.

As a point of general interest, the observations on mitoses confirm earlier observations that, whenever the L-1 agent has been involved in *Xenopus*, hematopoietic tissue of both the liver and spleen respond by proliferating. Cells in *Xenopus* liver did not respond when the normal allografts were made (L. N. Ruben and J. M. Stevens, unpublished) or when fetal calf serum used as an immunogen (L. N. Ruben, S. Bieber, and J. M. Stevens, unpublished). Further, *Xenopus* liver fragments will not respond immunologically to produce hemagglutinins to sheep or mouse erythrocytes in vitro (R. Auerbach and L. N. Ruben, unpublished). Results with spleen are available (1). It would appear then that, while the hematopoietic elements of the liver subcapsular epithelium fail to respond to immunogenic stimulation, they will respond to the presence of the L-1 agent by proliferating.

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REFERENCES

Amphibian Granuloma and Lymphoid Neoplasia


Fig. 1. Newt early L-1 focus. The principal cell type is epithelioid (h) with a peripheral areola of small lymphocytes (l). H & E, X 250.

Fig. 2. A typical growing L-1 focus in the newt is predominantly epithelioid in composition. H & E, X 250.

Fig. 3. The cellular composition in a newt granuloma is about 50% histiocytic (h) and 50% polymorphonucleocyte (p). H & E, X 400.

Fig. 4. A granuloma focus in the newt showing compression of neighboring liver epithelium with flattened cells (l) on the periphery. Note that the eosinophilic cytoplasm is more pronounced in granulomatosis. H & E, X 250.

Fig. 5. A large newt granuloma focus with some central caseation (c) and involvement of a giant, multinucleate macrophage (g). H & E, X 100.

Fig. 6. Newt transitional (?) state showing increase in mitoses (m) with shift toward a higher proportion of histiocytes to polymorphonucleocytes. The distinct eosinophilic cytoplasm is still apparent. H & E, X 250.

Fig. 7. Transitional (?) granuloma focus of the newt showing a larger proportion of histiocytes (h) to polymorphonucleocytes (p) to be compared with Fig. 3. Note the acid-fast bacteria (b) which are predominantly intracellular. Ziehl's carbol-fuchsin and hematoxylin, X 400.

Fig. 8. The cellular composition of a granuloma nodule of *Xenopus* is also about equally divided between histiocytes (h) and polymorphonucleocytes (p). H & E, X 500.

Fig. 9. Distortion of normal liver epithelium (d) by a granuloma nodule in *Xenopus*. H & E, X 250.

Fig. 10. Infiltration (i) of adjacent liver tissue by a transitional (?) focus in *Xenopus*. The liver epithelium is not distorted by its enlargement. H & E, X 250.

Fig. 11. The cellular composition of a *Xenopus* transitional (?) nodule shows a higher proportion of histiocytes with prominent nucleoli and nuclear membranes. Three mitoses are visible in this plane of focus. H & E, X 450.

Fig. 12. Macroscopic observations of liver from thymectomized (T) and nonthymectomized (N) *Xenopus* show many more white granuloma foci (f) visible after 21 days. H & E, X 40.
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