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N. F. Metcalf

S. P. Youngberg

W. K. Metcalf

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N. F. METCALF, S. P. YOUNGBERG and W. K. METCALF[1]

Abstract. Single cell suspensions of lymphocytes derived from the spleen and thoracic duct of rats were labelled with the fluorochrome acridine orange, proved to be viable by tissue culture, and injected into the left ventricle of isogenic rats. No evidence for recirculation of lymphocytes from blood to thoracic duct lymph within a three hour cycle was obtained. The injected cells whether derived from the spleen or thoracic duct homed only to the tissues of the lymphomyeloid complex.

The morphologically defined small round cell, the lymphocyte, almost certainly includes a number of populations of cells each with a different although perhaps related function. They can be found at many sites throughout the body including the blood, lymph nodes, spleen and bone marrow. In the rat, approximately 7×10^8 such cells are poured into the blood via the thoracic duct each day (Keohane and Metcalf, 1958), enough to replace the peripheral blood population tenfold in each 24 hours. As there does not appear to be sufficient cell division to account for the production of such large numbers of cells (Gowans, 1959b; Caffrey et al., 1962) and as it is difficult to believe that such large numbers can be destroyed each day (Gowans and Knight, 1964) without leaving some detectable evidence, recirculation of at least some of these cells a number of times from blood to lymph and back is implicit. Furthermore, prolonged drainage of the thoracic duct without reinfusion of the living cells so removed leads to a profound fall in thoracic duct cell output (Mann and Higgins, 1950; Gowans, 1957; Fish et al., 1969). Direct evidence for such recirculation, i.e., the direct identification of recirculating cells rests on two kinds of evidence, the autoradiographic identification of cells tagged with tritiated thymidine (Osmond and Everett, 1962; Everett et al., 1960; Rieke et al., 1960; Gowans and Knight, 1964) and the identification of fluorescence in cells labelled with a fluorochrome dye (Farr, 1951; Keohane and Metcalf, 1958). Autoradiographic investigations undoubtedly demonstrate recirculation of the label but the possibility of reutilization (Rieke, 1962; Bryant, 1962, 1963) and the problems of random pseudolabelling (Moffatt and Metcalf, 1970; Moffatt et al., 1970) casts some doubt on this evidence. We, therefore, decided to reinvestigate the possibility of using the fluorochrome acridine orange (De Bruyn

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et al., 1950) to demonstrate lymphocyte recirculation. It was also thought possible that the recirculation characteristics of lymphocytes from different sources may serve to give some indication of their relationship to one another.

Materials and Methods

The animals used were a highly inbred strain (at least 20 generations brother/sister mating) originally derived from the Worcester substrain of the Lister hooded rats. In all cell transfer operations litter mates were used and these were therefore essentially isogenic situations. Thoracic duct cannulation was performed by the method of Reinhardt (1946) under nembutal anesthesia. Single cell suspensions for injection or for monitoring were made by fine mincing in saline with scissors, allowing any large pieces to settle and decanting the cell bearing supernatant, repeating as necessary. Acridine orange supravital staining was carried out using a large volume of the appropriate solution of the dye in saline. The labelled cell suspensions were slowly injected into the left ventricle with a 20 gauge needle. Detection of acridine orange labelled cells was carried out with a Leitz combined phase and fluorescent microscope. This uses top illumination for fluorescence and transmitted illumination for phase so that it is possible to view the same field by fluorescence and phase either simultaneously or sequentially, at will. The culture technique is that described by Metcalf (1970). Cultures were repeated on at least four occasions with appropriate unstained controls.

Results

Effects of dye concentration on cell viability. Single cell suspensions derived from the spleen were stained with acridine orange and cultured both with and without phytohemagglutinin (PHA) for 72 hours at 37° C. With dye concentrations of 1/40,000 the cells, while still fluorescent at the end of 72 hrs., were much reduced in number and showed obvious signs of degeneration. There was some diminution in numbers at half the above dye concentration and many cells showed granules in their cytoplasm suggesting an attempt to excrete the dye. PHA cultures, however, showed many typical blastoid cells and numerous mitoses. When the dye concentration was further lowered to $1/10^6$ stained cells could not be differentiated from unstained cells by their culture characteristics. Fluorescent microscopy, however, showed that they still exhibited nuclear fluorescence with but little diminution in brightness. No unstained cells coud be detected.

Thoracic duct studies. In control experiments output of cells from a cervical thoracic duct fistula in fifteen anesthetized animals

was monitored at half-hourly intervals for up to 5 hours. The initial mean cell count of 19,000/cmm is somewhat lower than that often reported. This we attributed to the freedom of our animals from pneumonia (Schooley and Kelly, 1962) as when we repeated the experiments with a strain infected with the subclinical disease (histopathologically proved), we obtained the usual figure. The cell output steadily fell from an initial figure of approximately $18x10^6$ cells per hour to approximately 1/4 of this number at the end of 5 hrs. Infusion of 10^8 thoracic duct lymphocytes stained with $1/10^6$ acridine orange from an isogenic donor into the left ventricle 2 hrs. after the establishment of the fistula led to no change in the cell output. However, injection of a similar number of stained spleen cells seemed to cause one hour delay in the curve (fig. 1).

At no time, however, in spite of the relatively large number of stained cells injected was more than the occasional fluorescent cell detected in the thoracic duct lymph monitored at 5 min. intervals for the 3 hrs. after injection.

Blood monitoring. Samples of buffy coat preparations of tail blood were examined every five minutes for the first hour after injection for the presence of labelled cells. At no time were large

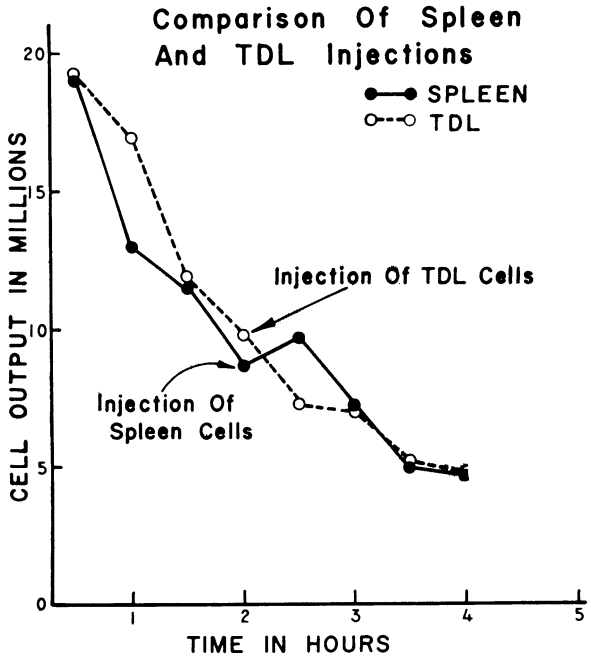

Fig. 1. The effect of the injection of 10^8 spleen or thoracic duct cells into the left ventricle of rats with a thoracic duct fistula on the ce'l output per hour. Each line is the mean of 12 experiments.

numbers of fluorescent cells found and in all cases the blood had been virtually cleared of them within one hour.

Tissue monitoring. Three hours after injection of fluorescent cells, they could be found in single cell suspensions of spleen, thymus, mesenteric and mediastinal lymph nodes, Peyer's patches of the small intestine and in the bone marrow. The problem of autofluorescence caused serious difficulties with quantitation but acridine orange labelled cells can be recognized by the difference in color of their fluorescence. Labelled cells were found only transiently in the lung, they were not present 3 hrs. after the injection, and they were not found at any time in the liver or kidney.

In the few experiments that were carried out with cells labelled with 1:40,000 acridine orange, labelled cells were found in blood and occasionally in thoracic duct lymph but in all cases they had been ingested by macrophages.

Discussion

From the experiments of Gowans (1959a) we would expect that the injection of so many cells into the vascular system would have led to some increase in the number leaving the thoracic duct. It is true that there does appear to be some increase when splenic cells were injected but this apparent increase is not statistically significant and in any case could account for no more than 15% of the injected cells. There would seem to be four possibilities: 1) The preparation of the single cell suspensions may have damaged the cells in some way—but they seemd to respond to culture normally and in any case this could not apply to the thoracic duct cells. 2) The conditions of the experiment were unphysiological in that the circulation was flooded with excessively large numbers of cells, but some surely should have recirculated normally under conditions when the thoracic duct output was low. 3) The circulation time is normally longer than the three hour duration of the experiment even though numerical calculation based on duct output and blood content has suggested a circulation time of 2 hours and the immediate fall in thoracic duct output following drainage aso suggests a rather short recirculation time. 4) The three hour drainage prior to injection of the cells had so depleted the central reservoir of cells, i.e., mainly the lymph nodes, that the injected cells were fully absorbed in replenishing this central reserve. Approximately 10^8 cells were lost to the animal in the preinjection and immediate postinjection period—roughly the same as the number of cells injected.

The absence of fluorescent cells from the thoracic duct is, of course, fully consistent with the numerical data discussed above. If they are so rapidly cleared from the blood and yet do not re-

appear in the thoracic duct lymph they must have either died and been destroyed, lost their label or be present in the tissues. The in vitro culture experiments strongly suggest that cells labelled with low concentrations of acridine orange ($1/10^6$) can retain the label for at least three days and do not seem to be injured although higher concentrations of dye are undoubtedly toxic (cf. Mimms, 1962). While the problem of enumerating the labelled cells in the tissues is complicated by the still incompletely described phenomenon of autofluorescence (Sainte-Marie, 1965a, 1965b) both injected spleen and thoracic duct cells homed onto all the components of the lymphomyeloid complex and were not detectable in other tissues. Uncertainty about the exact number of lymphocytes in each of the components of this complex in the normal animal and difficulties in determining the exact percentage of the labelled cells found in single cell suspensions prepared from tissues (1-2%) makes it difficult to determine what proportion of the injected cells can be accounted for in this way. Our findings confirm with splenic and thoracic duct cells the distribution in tissues of injected acridine orange stained lymph node lymphocytes (Farr, 1951; Rankin, 1960).

Conclusions

1. Neither our numerical or fluorescent tracer studies have provided any evidence for recirculation of small lymphocytes with a cycle time of less than three hours.

2. It has not been possible to differentiate splenic from thoracic duct lymphocytes on the basis of their ability to recirculate or on their homing propensities within the parameters of our experiments.

3. While acridine orange is a nontoxic and nonfading in vitro dye when used at suitable concentrations, its permanence in vivo has not yet been established.

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