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CELLULAR COMPOSITION OF LYMPH IN EXPERIMENTAL LYMPHEDEMA

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ABSTRACT

Lymph cell populations were characterized immunologically in dogs with chronic post-surgical lymphedema. There was a 10- to 30-fold increase in lymph total cell count as compared with normal (control) dogs. Morphologically, these were predominantly small lymphocytes with approximately 4-6% monocytes and veiled cells. In contrast, lymph from normal dogs contained 33% granulocytes and 27% monocytes. The Fc-R^+ and C3b-R^+ mononuclear nonadherent cells were less represented in lymph in chronic stasis than in normals. A high autotransformation rate of lymph cells and marked responsiveness to PHA and Con A were also observed. In chronic lymphedema K-cell and NK-cell cytotoxicity were lower than in normal lymph. Interruption or retardation of the lymphocyte recirculation pathway with lymph stasis may alter regulation of immune responsiveness in lymphedematous tissue.

Extensive division of peripheral lymphatics or wide removal of lymph nodes often induces permanent lymph stasis. Obstruction of lymphatics is also accompanied by recurrent bouts of lymphangitis, usually of streptococcal origin (1). Prolonged survival of skin allografts placed in areas deprived of lymph nodes (2) or with interrupted lymphatics (3-7) also has been observed. Total afferent lymphatic interruption in guinea pigs, moreover, allows temporary tumor allograft growth from a tumor cell inoculum that is ordinarily rejected when placed in an area with intact lymphatic drainage (8). Finally, the incidence of lymphangiosarcoma is higher in regions with chronic lymph stasis (9). Together, these findings suggest that in tissues deprived of normal lymphatic circulation immune surveillance is impaired. This deficiency may at least in part be related to a disturbed lymphocyte recirculation for lymphedematous tissue (10). Indeed, experimentally, lymph nodes in the area of lymph stasis undergo atrophy (11). Whether this phenomenon derives from impaired lymphocyte recirculation through regional nodes or from recurrent septic inflammation is, however, unclear.

Lymphocytes which become trapped in tissues with lymph stasis may undergo functional changes due to aging, or are influenced by environmental and tissue antigens and metabolites that induce relative immune insufficiency. Whereas a number of studies on the cellular composition of afferent lymph have been carried out in normal human (12-14) and animal (15-19) lymph, none have characterized immune cells in stagnant lymph. The present study, therefore, investigates changes in lymph cell subpopulations, their responsiveness to mitogens, and their cytotoxic activity in limbs with chronic lymphedema.

MATERIALS AND METHODS

Experimental groups

Thirteen mongrel dogs weighing 14-18kg were used. They were divided into 3 groups. In group 1 (*chronic* lymph stasis) (3 dogs) and group 2 (*acute* stasis) (2 dogs) surgical interruption of hind limb afferent lymphatics was carried out according to the method of Olszewski (20). Group 3 (5 normal dogs) served as controls. Studies in group 1 were carried out 5 years after induction of lymph stasis and that in group 2, 10 to 21 days after interruption of hind limb lymphatics.

Collection of lymph

In groups 2 and 3 lymph was obtained after cannulation of regional lymphatics. Lymph was collected continuously for 24h into syringes containing 0.2ml MEM medium with penicillin (100U/ml) and heparin (20U/ml). In group 1 lymph was collected by direct percutaneous puncture of regional dilated lymphatics.

Separation of lymph and blood cells

Lymph cells were separated on the Lymphoprep gradient (Nyegaard, Oslo). Mechanically defibrinated venous blood was diluted 1:3 with 0.9% NaCl solution, centrifuged on Lymphoprep gradient and washed 3 times with Hanks' medium.

Non-adherent cells isolation

Adherent cells were removed by incubation of the cell suspension in Petri dishes for 2h under culture conditions (RPMI 1640 medium supplemented with 20% fetal calf serum and 25mM HEPES). Non-adherent cells were recovered by washing.

Morphological characterization

Cell smears from centrifuge were stained by May-Grunwald-Giemsa method.

EA- and EAC-rosette forming cell assays

Sheep red blood cells (SRBC) were coated with anti-SRBC IgG (Cordis) or IgM (Cordis), respectively. Equal volumes of 5% SRBC suspension and 1ml of antibody solution were incubated for 30 min at 37°C. After washing, SRBC coated with IgM were suspended in 1ml of Hanks' medium and mixed with 1ml of 1:10 solution of AKR mice serum as a complement source. Incubation was carried out at 37°C for 30 min and after washing SRBC were subjected to 0.5% concentration in Hanks' medium. For tests 0.1ml of EA or EAC reagents were mixed with 0.1ml of lymphocytes (10^6 cells/ml), respectively. Samples were spun down for 2 min at 600rpm and incubated at 37°C for 30 min.

Blastic transformation with mitogens

Separated on Lymphoprep gradient cells were suspended in RPMI 1640 supplemented with 2mM L-glutamine, 100U penicillin, and 100μg streptomycin per 1ml and with 20% fetal calf serum. Cells were cultured in microculture plates at a density of $3x10^5$ cells/well in 0.2ml culture medium with or without mitogens. PHA (Wellcome) was used in the optimal concentration of 10μl/ml and Con A (Sigma) of 5μg/ml. Each test was performed in triplicate. Twenty hours before the termination of 72h culture each culture was labeled with 0.4 μCi of ^{3}H-thymidine (Amersham, 5 Ci/mM). Incorporation of radioactivity was measured on glass fiber filters in Permafluor cocktail (Packard).

NK-cell activity against K 562 cells

Target cells were labeled with $Na_2{}^{51}CrO_4$ ($3x10^6$ cells/ml with 100 Ci ^{51}Cr were incubated for 90 min). In test 100μl of target cell suspension (10^5) and 100μl of effector cells in different cell concentrations (40:1, 20:1, 10:1, 5:1) were mixed and incubated for 18h. Specific ^{51}Cr radioactivity release was measured in 100μl of supernatants.

K-cell activity against coated chicken red blood cells (CRBC)

CRBC were labeled with $Na_2{}^{51}CrO_4$ ($2x10^7$ cells/2ml with 100μCi ^{51}Cr) during 1h incubation. For tests 100ul of target cells (10^5) and 100μl of effector cells in different concentrations (2:1, 1:1, 1:2, 1:4) were mixed with 100μl of rabbit anti-CRBC serum (Cappel). After 18h incubation a specific release of ^{51}Cr radioactivity was measured in 100μl of supernatants.

Statistics

Comparisons were made by paired Student's t test and by analysis of variance.

RESULTS

Cell counts

The white cell count in afferent lymph of normal dogs was $0.23 \pm 0.21 \times 10^3$, in acute lymphedema $0.13 \pm 0.08 \times 10^3$, and in chronic lymphedema $4.7 \pm 3.8 \times 10^3$ cells/μl (chronic vs acute and normal $p < 0.02$). Evaluation of lymph cells smears revealed the presence of $90.5 \pm 3.0\%$ of lymphocytes, $3.6 \pm 2.5\%$ of monocytes and veiled cells, and $4.1 \pm 3.5\%$ of granulocytes in chronic lymph stasis vs $39.7 \pm 15.0\%$, $27.1 \pm 9.3\%$, and $33.1 \pm 17.4\%$ in normal lymph, respectively ($p < 0.001$) (*Fig. 1*).

There were no significant differences in cell percentage between normal and acute lymphedema lymphs.

After elimination of adherent cells the percentage of lymphocytes in lymph from chronic stasis did not increase significantly which was due to a low number of adherent monocytes and veiled cells. However, in normal and acute stasis lymph the percentage of lymphocytes rose by 11% and 22%, respectively. In the latter group the percentage of monocytes decreased from 29.4% to 9.0%.

Cells with Fc and C3b receptors

The percentage of EA-rosette forming cells (with Fc receptor) was $5.0 \pm 2.5\%$ in chronically stagnant lymph, $18.2 \pm 4.5\%$ in acute stasis, and $13.1 \pm 8.1\%$ in normal lymph

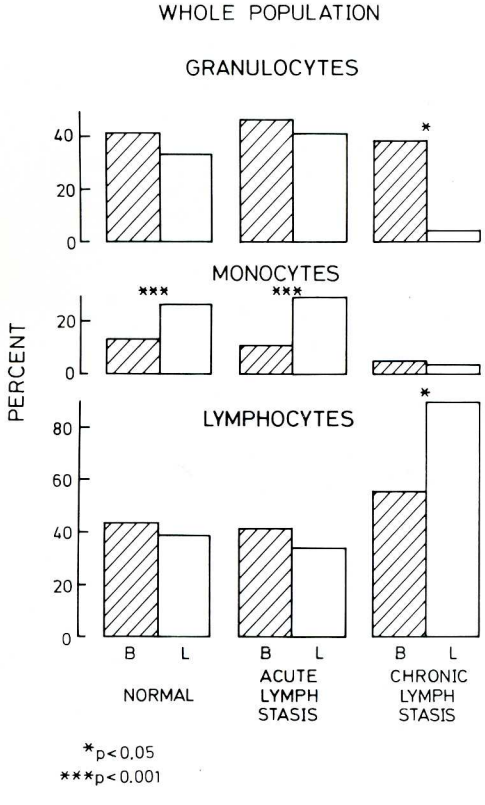

Fig. 1: Mean white cell counts in peripheral lymph (open bars) and blood (striated bars). Note increased lymphocytes and decreased monocytes in lymph with chronic stasis.

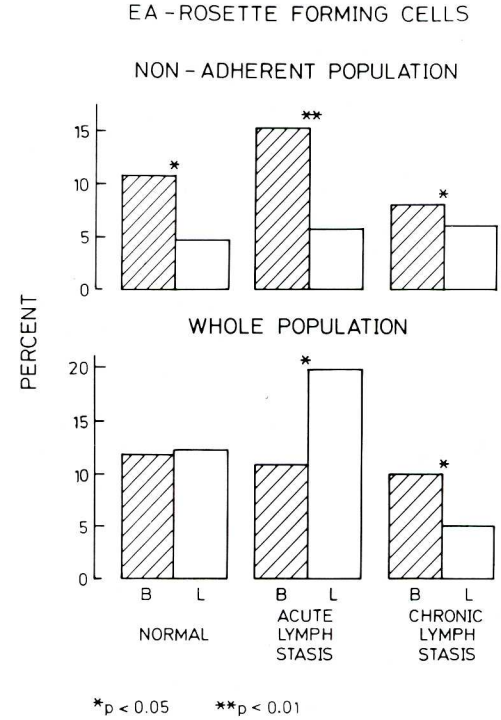

Fig. 2: Mean percentage of Fc-receptor bearing mononuclear cells in lymph (open bars) and blood (striated bars). Lowest values are in chronically stagnant lymph with most Fc-R$^+$ cells in the non-adherent fraction. In normal and acute stasis lymph the majority of Fc-R$^+$ cells are in the adherent subset.

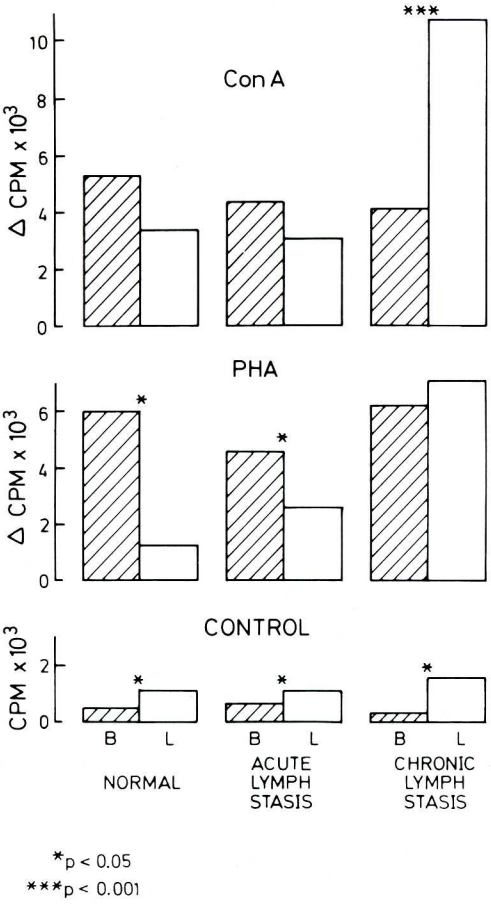

Fig. 3: Responsiveness of lymph (open bars) and blood (striated bars) cells to mitogens. Spontaneous blastogenesis higher in lymph than in blood in each group. Note that only in the chronic lymph stasis is the responsiveness of lymph cells higher than of blood cells.

(Fig. 2). The differences between chronic stasis and normal lymph were significant ($p < 0.01$). In the nonadherent population these percentages decreased to 6.0 ± 2.3%, 6.1 ± 4.0%, and 4.9 ± 3.2% respectively and the differences between all groups disappeared. This finding suggests that after elimination of monocytes only Fc-R$^+$ veiled cells and lymphocytes remained in the samples.

Similarly as Fc-R$^+$ cells, the EAC-rosette forming cells (C3b receptor) were less represented in lymph from chronic than

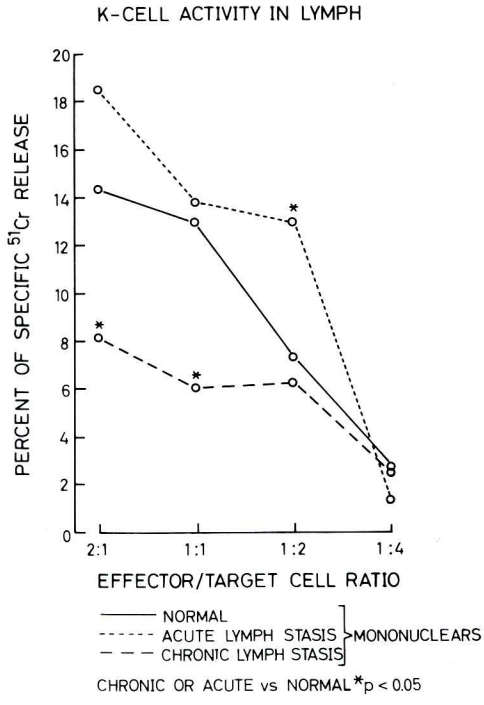

Fig. 4: The K-cell cytotoxic activity in lymph demonstrating the lowest values are in chronically stagnant lymph, a finding that coincides with lower number of cells with Fc-receptor in this group.

acute stasis or normal animals. The values were 3.5 ± 1.5%, 19.5 ± 10.5%, and 13.5 ± 7.5% respectively.

Responsiveness of lymph cells to mitogens

A significantly higher spontaneous blastogenic transformation rate was observed in lymphs from all groups than in blood ($p < 0.05$) *(Fig. 3).* Moreover, the reactivity of lymph cells to PHA was lower than to Con A in contrast to blood cells. The mean PHA/Con A ratio for lymph cells was 0.35 in normal dogs, 0.59 in acute lymph stasis, and 0.64 in chronic lymphedema, while for blood cells it was 1.11, 1.48, and 1.52 respectively. The responsiveness of lymph cells from chronic lymphedema to PHA and Con A was significantly higher than from normal lymph or acute lymph stasis ($p < 0.001$) *(Fig. 3).* Blood cells response to PHA and Con A was similar in each group.

Cytotoxic activity of lymph cells

The cytotoxic activity of K cells was lower in lymph from chronic stasis at E/T ratio 2:1 and 1:1 than in acute stasis and normals ($p < 0.05$) (*Fig. 4*). The NK-cell activity towards K562 cells was significantly lower in the cell population from chronic stasis than from other groups ($p < 0.001$) (*Fig. 5*). This was evident at E/T ratio 40:1, at other ratios the level of cytotoxicity remained in the range of laboratory error. The NK and K cells activity studied in blood samples was similar.

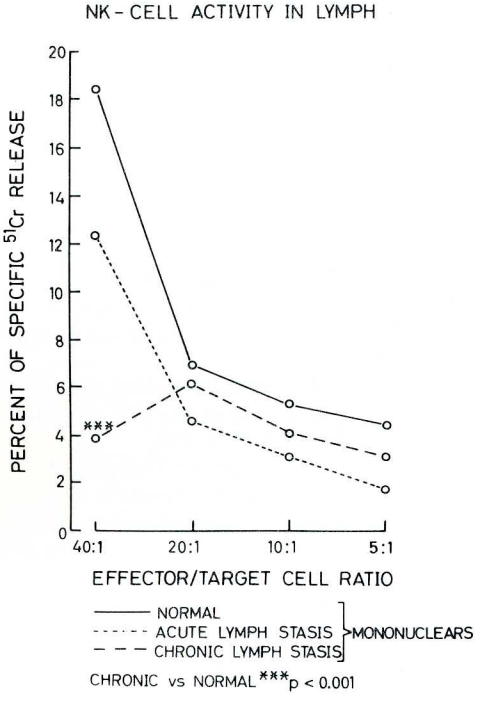

Fig. 5: NK-cell cytotoxic activity in lymph showing the lowest values are found in chronic lymphedema.

DISCUSSION

This study yielded the following information concerning cell composition in prenodal lymph in chronic postsurgical lymphedema in dogs: 1) a 10- to 30- fold increase in cell concentration compared with normal lymph, 2) change in distribution of subpopulations with decrease of granulocyte and monocyte concentrations and enrichment in lymphocytes, 3) low numbers of cells with Fc and C3b receptors, 4) high responsiveness to PHA and Con A with a high Con A/PHA ratio, 5) low level of K- and NK-cell cytotoxicity.

Normally, there is a low white cell count in afferent lymph, as compared with peripheral blood in humans (21) and animals (22) due to restricted extravasation from blood capillaries. Blockage of lymph outflow by interrupting the continuity of lymphatics promotes a several-fold rise in cell count. The observed accumulation of leukocytes in lymph in chronic lymphedema may be explained by impaired tissue fluid drainage and continuous migration of leukocytes from blood capillaries to tissues and accumulation in afferent lymphatics. Because a high rate of autotransformation is observed the possibility of *in vivo* cell proliferation in chronic lymphedema is also possible.

Lymph from "chronic stasis" , in contrast to "normal" or "acute stasis" is characterized by low percentages of monocytes and granulocytes. This finding may derive from selective inhibition of extravasation of these cells and restricted migration through the tissues with lymph stasis as they are not part of the recirculating pool of leukocytes. The percentage of veiled cells is slightly higher in the group with chronic stasis but other "differences" are not statistically significant.

The presence of granulocytes in afferent lymph is fairly specific for animals (23); in humans neutrophils are observed only sporadically (24). The "physiological" infection of the animal paw skin probably explains this difference in granulocyte extravasation.

Elimination of the adherent cells from lymph from chronic stasis did not produce major changes in the proportions of cellular subsets. A large portion of monocyte-like looking cells remained in suspension; veiled cells behaved in a similar manner. Moreover, neither of these macrophage-like cells phagocytized latex particles or a test strain of Streptococcus. We made similar observations in human lymph (12). These findings question the efficacy of morphologic evaluation of monocytes in lymph. In lymph from acute

stasis or normals, a large proportion of monocyte-like cells adhered to plastic, remained in suspension and did not phagocytize.

In the entire population of lymph cells, the percentage of cells with Fc-R and C3B-R was lowest in chronic stasis. Low occurrence of these receptors necessary for antibody-mediated cytotoxicity may account for the high propensity to develop infectious complications in lymphedematous tissues. Elimination of adherent cells evidently depleted normal and acute stasis lymph of cells with Fc-R. Nonetheless, in each group some 4-7% of FcR^+cells remained in suspension. They probably represent veiled cells, non- adherent monocytes and activated T-cells.

A higher spontaneous blastogenesis in lymph cells than in blood cells are found in each group with the highest values occurring in chronic lymph stasis. Moreover, the responsiveness to mitogens is unusually high in this latter group. Similar observations have been made in normal human prenodal lymph (14). There are several reasons for these observations: 1) a higher percentage of T-lymphocytes in chronic stasis as compared with acute stasis and normal lymph, and thus more cells with DNA-synthesizing capabilities, 2) an *in vivo* stimulation by bacterial, viral or intrinsic antigens persisting in stagnant lymph, 3) presence in stagnant lymph of veiled cells with potent antigen-presenting properties facilitating stimulation of lymphocytes. Thus, two additive stimuli (unknown intrinsic and extrinsic mitogenic) may be responsible for the high level of *in vitro* reaction. A higher responsiveness of lymph cells than of blood cells to Con A than to PHA most likely derives from immature populations of cells in lymph (25-27).

In chronic stasis, K-cell cytotoxicity is lower than in lymph from other groups, which most likely relates to lower numbers of cells with Fc receptor. The NK-cell activity is also lower, however, and because K562 target cells are relatively resistant to dog NK-cells, the results are difficult to interpret.

In summary, chronic lymph stasis following surgical interruption of lymphatics is accompanied by changes in lymph cellular composition: increase in total cell count, responsiveness to mitogens—but decreased cytotoxicity. Taken together these findings suggest that stagnant lymph cells remain active in the afferent arc of immune reactivity but are less effective in efferent responsiveness.

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