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The Effect of Steroids on the Circulating Lymphocyte Population

V. Effect of prednisolone treatment on cell size and life span of the thoracic duct lymphocyte population in normal and neonatally thymectomized rats. – A radioisotope study

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Summary

Using 3H-TdR isotope labelling in vivo, the effect of a high corticosteroid dose was correlated to cell size and life span of thoracic duct lymphocytes in rats.

The lymphocytopenia in thoracic duct lymph during the involution phase (3 hrs after treatment) was a marked depletion of small lymphocytes, followed by a restitution to pretreatment cell levels within one day. The original lymphocyte population had a higher per cent of labelled small lymphocytes (50 %) in comparison to the returning population which contained fewer (35 %) small labelled cells. Label index (i.e. labelled cells of a certain cell size in percent of total cell number) was unchanged for all cell sizes during both involution and restitution phases.

This unchanged label index profile as well as an earlier described unchanged cell size distribution support the hypothesis of lymphocyte trapping and redistribution as a major effect of a single prednisolone dose. The decrease of labelled small lymphocytes in the returning cell population can, however, also agree with a minor lymphocytolytic effect on circulating small lymphocytes.

Our data do not support the hypothesis of two different lymphocyte populations with different life span. Neither could a redistribution of lymphocytes be found in any lymphoid tissue compartment or in femur bone marrow, during the involution phase after corticosteroid treatment.

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Introduction

Since the recirculation of lymphocytes was described (1, 2), the problems of lymphocyte origin, migration, routes of recirculation, life span and cell size have been extensively investigated (see review by *Parrott* et al.) (3). Methods using incorporation of radioactive compounds have been widely used to study these aspects (4, 5).

The two main lymphocyte populations, derived from thymus and bone marrow, migrate different ways (6, 7, 8), with T-lymphocytes destined mostly for so-called thymus dependent areas and B-lymphocytes, preferentially for thymus independent areas of secondary lymphoid tissue (9).

Thoracic duct lymphocytes enter the lymph nodes from blood by migrating across endothelial walls of post-capillary venules and leaving via medullary region and efferent lymph vessels (10).

The life span of lymphocytes has been discussed and two different populations have been postulated, one so-called short lived and one long lived (11). According to *Everett* et al., the short lived population consists of cells that acquire isotope label within 4–5 days, while most of the long lived cells remain unlabelled (12). Estimation of the life span is less than 2 weeks for short lived and more than 2 months for long lived (13). Other works, however, show different average life span (14, 15, 16). The percentage of short

lived cells is calculated from data presented by *Everett* et al. and found to be in bone marrow and thymus almost 100%, in spleen 70–80%, in lymph nodes 20–30% and in blood 30–40% (11). Most thoracic duct lymphocytes were regarded as long lived but about 10% were supposed to be short lived. The short lived thymus derived small lymphocytes were found to migrate to bone marrow and spleen, while long lived small lymphocytes in the recirculating thoracic duct cell population, migrate to lymph nodes (17).

Some attempts have been made to correlate life span and lymphocyte function. A short lived cell was supposed to induce the hemolysine response, while a long lived cell seemed to produce antibodies (18). Lymphocytes with helper function are reported in two different T-lymphocyte populations (T_1 and T_2) (19). The T_1 helper cell seemed to be short lived and to help in the DTH reaction. Lymphocytes in the T_2 population were recirculating long lived cells with memory and helper function in the humoral response (20, 21). Long lived lymphocytes were found to accumulate in an experimental tumor but no certain cell function has been described for these cells (22).

Circulating lymphocytes are often divided in small, medium and large (11, 23), but no generally defined cell diameters have been stated for the different sizes. With a cells diameter of less than 7.5 μ the thoracic duct lymph contains 90 % small lymphocytes. Present knowledge of the capacity for blast transformation, found in small lymphocytes after antigen or mitogen stimulation, shows that cell size reflects the functional activity of the cell.

In relation to actual knowledge of immune functions, the effect of corticosteroids on the effector mechanisms is rather unknown. According to the lymphocytolytic effect on lymphoid tissue, mammals are regarded as either steroid-sensitive or steroid-resistant (24). Rat and mouse are sensitive, while humans seem to be more resistant. Our earlier work indicates that the lymphocytopenia seen in the circulation of a corticosteroid-sensitive species after prednisolone treatment is mainly a trapping and redistribution of cells (25).

The aim of this study is an attempt to define the circulating lymphocyte population according to life span and cell size and to correlate that to steroid-sensitivity. Using an isotope (3H-TdR) incorporation method, the thoracic duct lymphocytes are analyzed before, during and after injection of a high prednisolone dose.

Material and Methods

A. Rats of the Sprague-Dawley strain were neonatally thymectomized as earlier described (25) and nonthymectomized animals served as controls. Totally 78 animals, aged 7–10 weeks, were injected intraperitoneally twice a day for 7 days with a dose of 50 μCi/100 g body weight of 3H-TdR (Thymidine methyl-3H, NET-027A 2 μCi/mmol. Sterile aqueous solution, New England Nuclear, Dreieich, West Germany). One group (the so-called short lived group) of animals was subjected to steroid treatment and thoracic duct drainage one day after the last isotope injection. The other group (the so-called long lived group) was examined in the same way 2 weeks later. A dose of 10 mg/100 g body weight of prednisolone sodium succinate (Precortalon Aquosum, Organon) was given intramuscularly. Three or 24 hours after injection a thoracic duct drainage was started (26). Smears of lymph and venous blood were prepared for autoradiography with a grain density method (27), using Ilford L4 Nuclear Emulsion. After exposure for 4–5 weeks the slides were developed and the lymphocytes measured according to label and cell size, using an eye-piece micrometer. Cells with a diameter less than 7.3 μ were regarded as small and cells with a diameter more than 9.7 μ as large lymphocytes.

B. Normal rats were injected intraperitoneally every third hour during 36 hours with the same dose of 3H-TdR as above. After thoracic duct drainage, lymph samples (10×10^6 cells) were infused intravenously in siblings, total 42 rats. Half of the recipient animals received a prednisolone injection 30 minutes prior to

Table 1 Cell count (labelled and unlabelled cells) in thoracic duct lymph in normal and neonatally thymectomized rats at different times after a corticosteroid injection (mean ± standard error of the mean)

	Cells /μl			
	Total	Small ($\phi < 7.3\ \mu$)	Medium (ϕ 7.3–9.7 μ)	Large ($\phi > 9.7\ \mu$)
Nonthymectomized:				
Untreated (n = 13)	42455 ± 3771	33239 ± 3915	7424 ± 2002	1469 ± 352
3 hours after steroid treatment (n = 14)	19177 ± 1646	13312 ± 1911	4689 ± 1191	1274 ± 365
24 hours after steroid treatment (n = 14)	36894 ± 4310	23500 ± 2228	11859 ± 3361	1540 ± 357
Thymectomized:				
Untreated (n = 12)	13030 ± 1030	9912 ± 1059	2222 ± 440	969 ± 193
3 hours after steroid treatment (n = 11)	4355 ± 965	3433 ± 964	638 ± 212	297 ± 115
24 hours after steroid treatment (n = 14)	10129 ± 1106	8355 ± 1002	1324 ± 432	512 ± 249

the lymph infusion. A thoracic duct drainage was started 1, 3, or 24 hours later. Lymph, venous blood, thymus, spleen, lymph nodes, femur bone marrow and liver were prepared for liquid scintillation by extraction of DNA with TCA at +90 C (28). The isotope activity was measured in a Packard Tri Carb beta-scintillator using Insta-Gel as scintillation liquid (Packard Instruments Co., Inc., Downers Grove, Ill., USA).

Cell counts are given as the mean and standard error of the mean and Student's t-test is used to compare the different groups.

Results

A. Total cell count in thoracic duct lymph 3 hrs (involution) and 24 hrs (restitution) after a corticosteroid injection was about the same as in earlier work (25), with a significant depletion of cells (55 %) followed by a return to pretreatment cell level (see Table 1 and Fig. 1). The per cent of labelled cells were about the same in both short and long lived groups (see Table 3). The short lived groups (normal and thymectomized animals) had 40–47 % of the lymphocytes labelled while a somewhat lower per cent, 30–45 %, was found in the long lived group. Cell counts for different cell sizes are, therefore, presented as one group for both short and long lived animals.

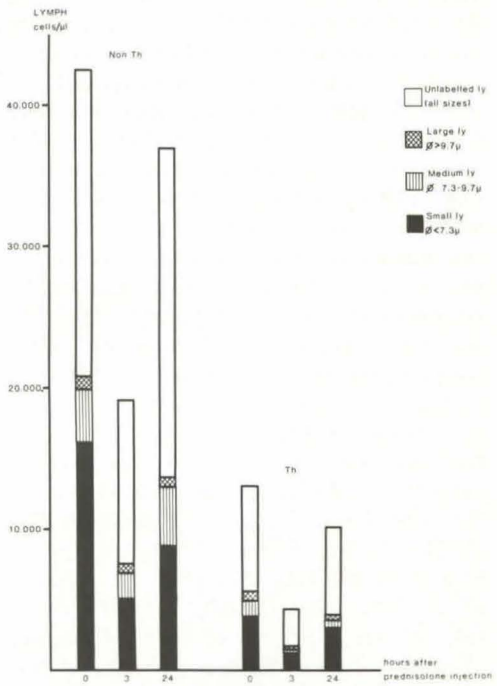

Fig. 1 Cell count of small, medium and large lymphocytes in thoracid duct lymph in normal and neonatally thymectomized rats at different times after a corticosteroid injection

Table 2 Distribution of small, medium and large lymphocytes (both labelled and unlabelled) in thoracic duct lymph in normal and neonatally thymectomized rats at different times after a prednisolone injection

	% Small ($\phi < 7.3\ \mu$)	% Medium (ϕ 7.3–9.7 μ)	% Large ($\phi > 9.7\ \mu$)
Nonthymectomized:			
Untreated (n = 13)	77	18	4
3 hours after steroid treatment (n = 14)	69	24	7
24 hours after steroid treatment (n = 14)	70	27	4
Thymectomized:			
Untreated (n = 12)	76	17	8
3 hours after steroid treatment (n = 11)	78	15	8
24 hours after steroid treatment (n = 14)	84	13	4

The depletion of thoracic duct lymphocytes after steroid treatment in normal and thymectomized animals, were significant only for the small lymphocytes (both labelled and unlabelled). The restitution of cell levels were significant in both normal and thymectomized animals (Table 1), but small labelled lymphocytes were only partly regained in normal animals (see Fig. 1). The returning cell population in this group was dominated by unlabelled small lymphocytes, which replaced the missing labelled cells. The returning lymphocyte population had 35% of the small lymphocytes labelled compared to 50% for the original population. Medium and large lymphocytes were not statistically different during the different phases after corticosteroid treatment. Percental distribution of thoracic duct small, medium and large lymphocytes (both labelled and unlabelled), were the same during the different phases (Table 2). Distribution of labelled lymphocytes in both short and long lived groups is presented as a label index (labelled cells of a certain cell size in percent of the total number of cells) as seen in Table 3. The label index did not change significantly during involution or restitution.

B. Within 24 hrs after the infusion of labelled lymphocytes in siblings, about 60% of the injected 3H-TdR activity could be detected in thymus, spleen, lymph nodes and liver (see Table 4). No increase in isotope activity could be found in any of these organs during the involution phase after prednisolone treatment. Femur bone marrow and thoracic duct lymph show very low isotope activity which makes the calculation somewhat hazardous. There were no differences in the values during the different phases after the steroid treatment. Estimated total venous blood volume contained about 10% of the injected activity during the different phases.

Discussion

Different radioisotopes can be used to label lymphoid cells (4), either incorporated in cell structures (e.g. 3H-TdR in vivo) or attached to the cell surface (e.g. Cr^{51} in vitro). It can not be excluded that the isotope can change the cell function by radiation (29). 3H-Thymidine is exclusively incorporated in the DNA of dividing cells. When administered twice daily, about half of the cell cycles are available for labelling. The amount of labelled cells depends on the length of time the isotope is available, but can also be influenced by reutilization of isotope which has been liberated from dying cells which were already labelled (30). The migration of lymphocytes is

Table 3 *Label index:* Labelled small, medium and large lymphocytes, in percent of all labelled and unlabelled cells in thoracic duct lymph in normal and neonatally thymectomized rats at different times after a corticosteroid injection

	"Short lived" lymphocytes				"Long lived" lymphocytes			
	% Small	% Medium	% Large	% Total	% Small	% Medium	% Large	% Total
Nonthymectomized:								
Untreated	41	4	2	47	31	12	2	45
3 hour after steroid treatment	32	10	6	47	26	9	2	36
24 hours after steroid treatment	27	11	2	40	26	6	2	34
Thymectomized:								
Untreated	29	9	5	44	29	8	4	41
3 hours after steroid treatment	33	6	6	45	19	8	4	31
24 hours after steroid treatment	32	7	4	43	25	4	1	30

Table 4 Per cent of isotope activity found in different organs in normal rats at different times after intravenous infusion of labelled lymphocytes without/with prednisolone

		Thymus	Spleen	Total lymph node tissue*	Liver
After lymphocyte infusion:					
1 hour	(n = 6)	3	17	18	20
3 hours	(n = 7)	1	13	21	17
24 hours	(n = 10)	1	7	33	13
After lymphocyte and corticosteroid infusion:					
1 hour	(n = 7)	2	13	12	13
3 hours	(n = 6)	1	10	12	11
24 hours	(n = 6)	1	6	21	19

very complex, as cells from different sources have different routes and destinations (3). Different isotopes can change the destination of cells from the same source, probably by labelling different lymphocyte populations (31). T- and B-lymphocytes migrate to different compartments of lymphoid tissue (9), but both cell types are found in the so-called gut-seeking population which migrates to lamina propria of the intestinal tract (32). No more than about 50% of thoracic duct lymphocytes can be labelled even with an isotope administration of 2 months (3). Based on data presented by *Everett* et al. (11, 12) we expected about 25% of short and 5% of long lived thoracic duct cells to be labelled with our administration scheme. The results show that about 45% of all cells were labelled in both normal and thymectomized animals and remained so also after 2 weeks. This high percentage of labelled circulating lymphocytes in thoracic duct lymph found both directly after the isotope administration and also 2 weeks later did not support the hypothesis of two different cell populations of which the short lived one has a life span less than

2 weeks. To divide the circulating lymphocytes into short and long lived populations, separated by a defined time limit, seems to be a very artificial subgrouping. Our results can be explained by assuming a larger lymphocyte population with a short life span. Definition of short and long life span varies in the literature and no proper border can be defined. It is not defined whether the life span ends when the cell is dividing or when the cell is dying. There is no information about different life span for T- and B-lymphocytes. Neither is it known if short and long lived cells are two distinctly separated lymphocyte populations or if the same cell type can be both short and long lived. It is impossible to estimate the total life span by determining the period of time that labelled cells remain in a lymphoid organ. The cells could have been circulating earlier or they may start to recirculate later. To discriminate between stationary lymphocytes in an organ and lymphocytes temporarily arrested during recirculation seems impossible. All small lymphocytes have the same appearance in the microscope but can have quite different cell functions. Two different T-lymphocyte populations (33) have been defined in mice according to life span and steroid sensitivity (19). Both populations were shown to contain cells with helper function (20, 21). A memory cell function was found in the long lived cell population. Spleen cells show during infection with Listeria monocytogenes two cell populations with different life span, different cell functions and different corticosteroid-sensitivity (34). The proliferating cells were short lived and steroid-sensitive while the other cell population was long lived and steroid-resistant. The steroid-sensitive and short lived cell can protect against infection when transferred, thus showing a specialized function. It is also reported that a long lived cell, after antigen stimulation and proliferation, produces new cells with a short life span (35). There are few convincing data that correlate a short or long life span to specialized cell functions. It seems likely that the memory cell function is correlated to long lived cells.

The corticosteroid effect on cells with different life span have been discussed. Long lived lymphocytes are regarded as resistant, while short lived are sensitive, as determined by the lytic effect of steroids (36, 37). In our work it is impossible to separate short lived from the long lived lymphocytes in thoracic duct lymph. When comparing the groups, no changes could be seen that indicate any difference in steroid-sensitivity of so-called short and long lived lymphocytes. The effect of neonatal thymectomy was the same as earlier described (38, 39), with a reduction of small circulating lymphocytes. Despite this reduction of small cells the thymectomized animals showed the same changes as normal animals concerning label index.

The returning lymphocyte population, after steroid induced involution, contained more unlabelled small lymphocytes than found in the original cell population (see Fig. 1). Some of the labelled cells have been replaced by unlabelled cells. This could be due to a lymphocytolytic effect or due tc a prolonged trapping It is known that thoracic duct lymphocytes in rat are sensitive to the lytic effect of corticosteroids in vitro (40) and we can not exclude a minor lymphocytolytic effect also in vivo.

It is not known, if a trapping mechanism is a steroid effect directly on the lymphocytes or on the tissues. An effect on the cell surface could perhaps change the migratory pattern of the lymphocytes. On the other hand, tissues exposed to corticosteroids might perhaps change their blood vessels, the connective tissue and the cells in the reticulo-endothelial system. These changes can probably prolonge the passage time for the circulating lymphocytes. There are no data in the literature really favouring either of these alternatives.

Earlier reported data with unchanged cell size distribution (41) and an unchanged distribution of T- and B-lymphocytes (*Hedman, Röckert* and *Lundin:* to be published) as well as the present data with unchanged label index profile of different cell sizes indicate a high degree of identity between the original, the disappearing and the returning cell populations. This identity agree with the hypothesis of lymphocyte trapping and redistribution as a major steroid effect on circulating lymphocytes. The anatomical localization of this trapping has not

been satisfactorily clarified. Both lymphoid tissue and bone marrow have been supposed to represent the anatomical compartment for the trapping mechanism, but no really convincing data have been presented. An increase in theta-antigen positive cells is reported in bone marrow in mice after steroid treatment (42, 43). However, according to actual knowledge, the theta-antigen is not specific for T-cells and can also be induced in bone marrow from theta-negative precursor cells (44, 45). Using Cr^{51} labelled lymphocytes, an accumulation in guinea pig bone marrow was shown after steroid treatment (46). Cr^{51}-labelling is a rather unphysiological method and the distribution of the isotope does not necessarily reflect the distribution of the living cells. We could not confirm any increase of 3H-TdR labelled lymphocytes (thoracic duct lymphocytes from siblings) in the femur bone marrow, lymphoid tissue or liver during the involution phase after steroid treatment. It seems possible that the trapping mechanism is not restricted to a localized anatomical compartment.

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