

## High Spontaneous and Mitogen-induced Activity of Mononuclear Cells in Lymph Draining Normal Human Skin

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### Summary

Lymphocytes in afferent lymph draining skin are derived from populations selected at the capillary level to enter the tissue space and which migrate spontaneously through the tissue to the initial lymphatics. These cells reveal a high spontaneous transformation rate in a 72 h culture. The mean  $^3\text{H}$  TdR incorporation was in a 24 h culture 2 times and in a 72 h culture 5 times higher than that of the peripheral blood mononuclear cells of the same subjects. The lymph cells responded significantly stronger to low concentrations of PHA, ConA and PWM than the blood cells. Also, the peak response of lymph cells was apparent at lower concentrations of mitogen in culture than of blood cells. The lymph cell population revealing a high spontaneous transformation rate was found to be the OKT4 enriched subset (induced/helper). The findings of a high spontaneous activation of lymphocytes which trafficked through the normal skin and defining this highly reactive subset as an OKT4-positive and strongly responsive to mitogens, reflect the *in vivo* immune events in the normal skin.

### Introduction

The process of immune surveillance in the skin is controlled by: a) recirculating lymphocytes, capable of recognizing altered self, aberrant and potentially malignant cells and virus-infected cells and eliminating them, and b) the antigen-processing epidermal Langerhans cells. These cells also play an important role in the antigenicity of skin in an allograft context as the "passenger" immunocompetent cells (10). When confronted by histoincompatible antigens they are able to proliferate and differentiate directly and *in situ* (12). The lymphoid cells migrate into the skin from the blood

and subsequently, at least in part, leave it via lymph vessels (8). There appears to be some selection of migrating cells at the level of the capillary bed by which only certain cell types are selected to enter the skin (3, 4, 6). A more precise defining of their specific assignments in the tissue requires further phenotypic and functional characterization. In a previous paper (7) we made use of monoclonal antibodies to characterize the cell populations which migrate into the normal human skin and which, having traversed the tissue, could be recovered from the afferent lymph vessels. Significant differences were apparent between the types and proportions of cell populations in lymph leaving skin and blood.

In this paper we present our findings on the autotransformation and responsiveness to mitogens of various subsets of mononuclear cells obtained from the skin — derived afferent lymph of normal men. The responsiveness of lymph and blood cells from the same subjects was compared.

### Material and Methods

**Experimental design:** The following parameters were measured: a) autotransformation rate of lymph and PBM cells after 4, 24 and 72 h culture *in vitro*, b) responsiveness of lymph and PBM cells to PHA, ConA and PWM, c) autotransformation rate and responsiveness to PHA and PBM cells cultured in medium supplemented with lymph, d) autotransformation rate and responsiveness to PHA of lymph and PBM cell OKT4<sup>+</sup> and OKT8<sup>+</sup> enriched populations.

Because lymph was collected through plastic cannulas into test-tubes for periods over 12 h, whereas PBM cells were collected over much shorter periods of time and with the use of syringes, control studies have been carried out in order to detect any possible effect on the responsiveness of peripheral blood lymphocytes of different storage periods and type of materials used for collection of samples.

*Lymph cell collection:* Lymph was collected from a leg superficial vessel. This vessel drained the skin, subcutaneous tissue and perimuscular fascia of the foot and part of the lower leg. The technique of lymphatic cannulation was described previously (3). Briefly, a lymph vessel running along the anterior aspect of the leg was dissected under sterile conditions and a polyethylene P60 Clay-Adams siliconized tapered cannula inserted into its lumen. The external tip of the cannula was placed into a sterile 10 ml plastic test-tube containing 1 ml of PBS with 20 units of heparin without preservatives. The volunteers were allowed to walk normally. Lymph mononuclear cells were obtained directly from lymph samples collected over periods of 6–12 h by centrifugation. Since the lymph cell population contained no granulocytes and only very few erythrocytes, a density gradient separation step was unnecessary.

*Blood mononuclear cell isolation:* Blood samples were taken from the cubital vein. Mononuclear cells were isolated by centrifugation on Lymphoprep (Nyegaard, Oslo) at 1600 rpm for 35 min. Cells from the interface were collected, washed three times in RPMI 1640, and adjusted to appropriate concentrations.

*Mitogen induced proliferation:* Lymphocytes were suspended in RPMI 1640 medium supplemented with 15% heat-inactivated foetal calf serum (Gibco) and 100 u/ml of penicillin-streptomycin solution. Triplicate cultures were prepared in flat-bottom microculture plates (Sterilin, Teddington, England) using 0.2 ml aliquots of suspension containing  $1 \times 10^5$  cells. PHA (Wellcome) was used at concentrations: 0.45, 1.8, 4.5, 18.0 and 90.0  $\mu\text{g/ml}$ , ConA (Sigma) at: 0.1, 0.5, 1.0, 2.0 and 5.0  $\mu\text{g/ml}$ , PWM (Gibco) at 0.1, 0.5, 1.0, 5.0 and 10.0  $\mu\text{g/ml}$ .

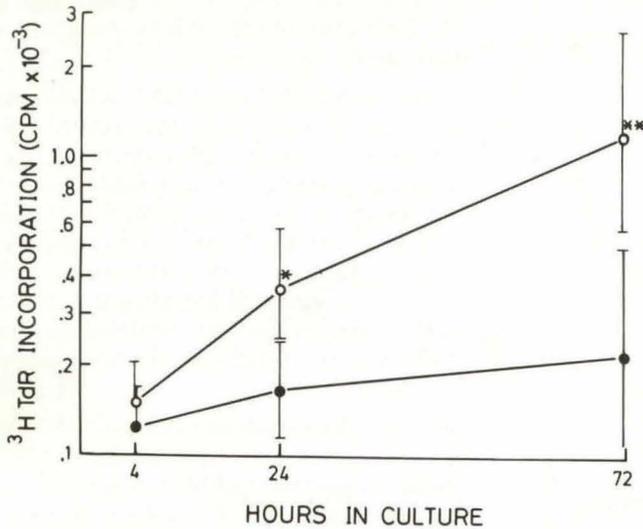
Culture plates were incubated for 3 days at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Twenty hours before harvesting 0.4  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine (spec. act. 5 Ci/mmol., Amersham, England) was added to each well. Cells were harvested using a Multimash harvester (Titertek, Flow Lab.). Control cultures without addition of mitogen, were included for each cell sample. Incorporation of radioactive thymidine was evaluated in a liquid scintillation counter.

*Isolation of lymphocyte subsets by complement-mediated lysis with the monoclonal antibodies OKT4 and OKT8.*

In order to isolate lymphocyte populations highly enriched in either OKT4 (helper/inducer) or OKT8 (suppressor/cytotoxic) reactive cells, 0.4 ml of cell suspension ( $2.5 \times 10^6$  cells/ml in RPMI 1640 medium with 5% fetal calf serum) was mixed with 50  $\mu\text{l}$  of OKT4 (2.5  $\mu\text{g}$ ) or OKT8 (5.0  $\mu\text{g}$ ) antibody (Ortho Diagnostic, Raritan, New Jersey). After incubation for 5 min at 37 °C, 50  $\mu\text{l}$  of fresh rabbit serum – as a source of complement – was added and cells were incubated for another 45 min at 37 °C. Subsequently, the cells were washed once with RPMI 1640 and the percent of viable cells was determined by the Trypan blue exclusion method. It was 49% and 65% in lymph and 51% and 48% in blood for cells treated with OKT4 and OKT8, respectively. We use the notation OKT4<sup>+</sup> to signify a population of T-cells remaining after treatment with OKT8 plus C and OKT8<sup>+</sup> to signify the reciprocal population of T-cells remaining after treatment with OKT4 plus C. The OKT4<sup>+</sup> or OKT8<sup>+</sup> lymphocytes were suspended in RPMI 1640 supplemented with 15% of heat-inactivated fetal calf serum (Gibco) and cultured in triplicate in flat-bottom microculture plates (Sterilin, England) using 0.2 ml aliquotes containing  $1 \times 10^5$  cell.

*Subjects:* Twelve healthy male volunteers, aged 18–26 years, were studied.

*Statistical methods:* Values were expressed as geometric means  $\pm$  standard deviation. For evaluation of the statistical significance of differences in results where a comparison between values for lymph and blood is made, the t-test for pairs was used.



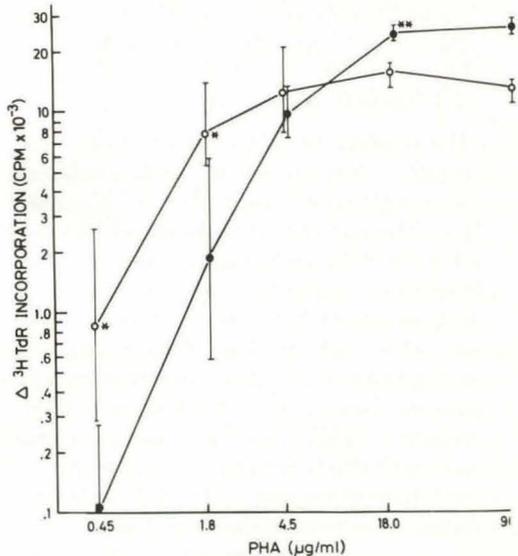
**Fig. 1** Spontaneous transformation rate of lymph (O) and PBM (●) cells.  $2 \times 10^5$  cells/ml cultured without mitogens. Values are geometric means  $\pm$  S.D. of 12 tests. \*Lymph vs blood  $p = 0.03$ , \*\*Lymph vs blood = 0.00003

**Results**

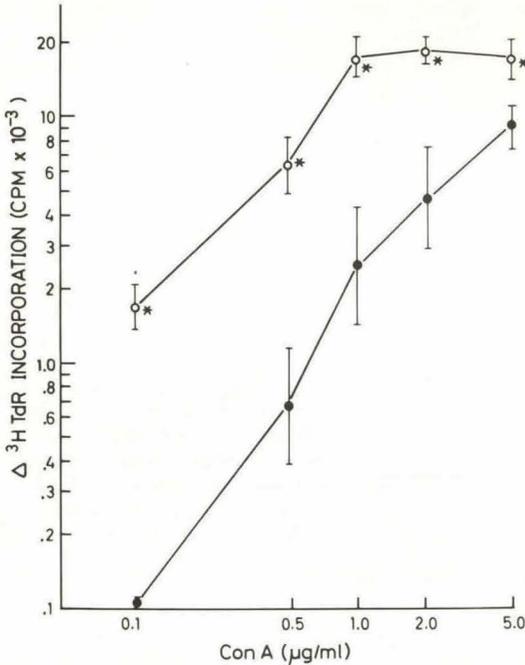
*Spontaneous transformation of lymph and PBM cells:* Lymph cells cultured without mitogens for periods 24 and 72 h revealed an increasing incorporation of [ $^3\text{H}$ ] TdR when compared with PBM cells (Fig. 1). The 24 h culture brought about an increase of [ $^3\text{H}$ ] TdR incorporation into lymph cells over the PBM cells with values  $363^{+225}_{-129}$  cpm and  $165^{+69}_{-48}$  cpm ( $p = 0.033$ ), respectively. After 72 h culturing, the values for lymph cells were  $1230^{+1524}_{-681}$  cpm, for PBM  $218^{+319}_{-129}$  cpm ( $p = 0.00003$ ).

*Activation of lymph and PBM cells by PHA, ConA and PWM:* The response curve of lymph cells to all three mitogens differed considerably from that of PBM cells. The concentration of PHA as low as  $0.45 \mu\text{g/ml}$  evidently stimulated lymph cells ( $870^{+1760}_{-582}$  cpm), whereas there was no response of the PBM cells to that dose (Fig. 2). At the concentration of PHA in the culture of  $1.8 \mu\text{g/ml}$ , the [ $^3\text{H}$ ]TdR incorporation in lymph cells was  $7943^{+6182}_{-2573}$  cpm, in PBM cells  $1862^{+4163}_{-1282}$  cpm ( $p = 0.024$ ). The peak response of lymph cells to PHA was reached at the concentration

of  $4.5 \mu\text{g/ml}$  of mitogen. The PBM cells response curve was, at low concentrations of PHA, below that of lymph cells. However, at



**Fig. 2** Activation of lymph (O) and PBM (●) cells with PHA.  $1 \times 10^6$  cells/ml cultured for 72 h. Values are geometric means  $\pm$  S.D. of 7 tests. \*Lymph vs blood  $p < 0.05$ , \*\*Blood vs lymph  $p < 0.05$



**Fig. 3** Activation of lymph (O) and PBM (●) cells with ConA.  $1 \times 10^6$  cells/ml cultured for 72 h. Values are geometric means  $\pm$  S.D. of 7 test. \*Lymph vs blood  $p < 0.05$

concentrations of 18.0  $\mu\text{g/ml}$  and 90  $\mu\text{l/ml}$  the PBM cell response curve reached a level high above that of lymph cells ( $p = 0.001$  and 0.00003, respectively).

The response of lymph cells to ConA was evidently high already at the concentration of mitogen of 0.1  $\mu\text{g/ml}$  ( $1698 \pm_{318}^{391}$  cpm), but there was still no activation of PBM cells (Fig. 3). With increasing concentrations of ConA the lymph cells responded much stronger than the PBM cells. At the concentrations of ConA of 2.0  $\mu\text{g/ml}$  the mean incorporation of [<sup>3</sup>H]TdR in the lymph cells reached a peak and was  $18620 \pm_{1025}^{2272}$  cpm whereas in PBM cells  $4677 \pm_{1726}^{2736}$  cpm. The statistically significant differences between lymph and PBM cell activation at the level of below 0.05 were found at all concentrations of ConA.

The activation with PWM was evident in lymph cells at the concentration of 0.1  $\mu\text{l/ml}$  ( $512 \pm_{339}^{1001}$  cpm) but not in the PBM cells ( $76 \pm_{41}^{20}$  cpm). The increasing concentrations of

PWM activated lymph cells slightly more than the PBM cells, but the differences were not statistically significant.

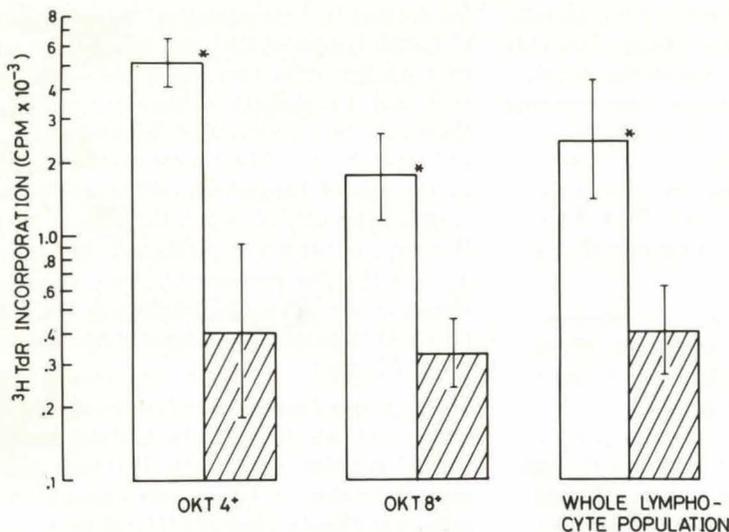
*Effect of tubing and test-tube material and collection procedures on activation of lymphocytes.* Culturing of PBM cells with fragments of polyethylene cannulas, as well as after storage in plastic test-tubes for 24 h in the culture media and lymph did not increase the autotransformation rate and response to PHA. Also, the response of Lymphoprep-isolated and cultured lymph cells remained unaltered to PHA as compared with the non-isolated population.

*Autotransformation rate and response to PHA of PBM cells cultures with wupplement of lymph.* Supplementing of culture media with lymph from 15 to 100% did not increase the spontaneous transformation rate and response to PHA of the PBM cells. Thus, the high-degree response of lymph cells could not be attributed to the presence of stimulating factors in lymph.

*Autotransformation rate and response to PHA of lymph and PBM cell OKT4<sup>+</sup> and OKT8<sup>+</sup> enriched populations.* The OKT3<sup>+</sup> enriched lymph cell subset revealed a high autotransformation rate after a 72 h culture ( $5128 \pm_{1055}^{1328}$  cpm) (Fig. 4). It was higher than of the whole population ( $2454 \pm_{1974}^{1911}$  cpm) ( $p < 0.05$ ) and of the OKT4<sup>+</sup> enriched PBM population ( $406 \pm_{223}^{526}$  cpm) ( $p = 0.013$ ). It was also significantly higher than the autotransformation rate of the lymph cell OKT8<sup>+</sup> enriched subset ( $1737 \pm_{575}^{893}$  cpm) ( $p < 0.05$ ). The [<sup>3</sup>H] TdR incorporation of OKT8<sup>+</sup> enriched population of lymph cells was higher than of the PBM cells ( $p < 0.05$ ), but lower than that of the whole unseparated lymph population.

Neither the OKT4<sup>+</sup> nor the OKT8<sup>+</sup> enriched populations of PBM cells revealed any rise of autotransformation rate over normal values.

Activation of the OKT4<sup>+</sup> enriched population of lymph cells with 1.8  $\mu\text{g/ml}$  of PHA resulted in a rise of [<sup>3</sup>H] TdR incorporation by  $1621 \pm_{930}^{2180}$  cpm over the controls. It was slightly higher than that of PBM cells ( $1202 \pm_{269}^{346}$  cpm), however, the difference was not statistically significant.



**Fig. 4** Spontaneous transformation rate of the OKT4<sup>+</sup> and OKT8<sup>+</sup> - enriched populations of lymph (open bars) and PBM (shaded bars) cells.  $1 \times 10^6$  cells/ml cultured for 72 h without mitogen. Values are geometric means  $\pm$  S.D. of 4 tests. \*Lymph vs blood  $p < 0.05$

The OKT8<sup>+</sup> enriched lymph cell population responded to PHA much stronger than the PBM population, 3890  $^{+2626}_{-1600}$  cpm and 398  $^{+262}_{-159}$  cpm, respectively ( $p = 0.011$ ).

The differences in response to PHA between the OKT4<sup>+</sup> and OKT8<sup>+</sup> enriched lymph cell populations were not statistically significant.

### Discussion

This study has demonstrated that: a) cells from normal skin-derived lymph, mostly T-lymphocytes, undergo a significant spontaneous transformation when cultured *in vitro*, whereas blood lymphocytes from the same subjects do not, b) lymph cells respond very actively to low concentrations of polyclonal mitogens, while the same concentrations remain substimulatory for the PBM cells, c) the population undergoing the high spontaneous transformation belongs to the OKT4<sup>+</sup> (inducer/helper) subset.

Thus, the question arises as to what is the mechanism of spontaneous stimulation of lymph lymphocytes and of their enhanced responsiveness to mitogens. The high auto-transformation rate of lymph cells observed in our studies during the first 24 h period of *in vitro* culture, may be attributed to the

factors stimulating lymphocytes in the tissues, as well as, during and after *ex vivo* collection. The priming of lymphocytes with "internal" or "environmental" antigen *in vivo* might have occurred when they traversed the extravascular space. No direct evidence for that has been found in the present study. However, our recent unpublished observations could indicate that the activation occurs prior to the *ex vivo* collection of lymph. We found that neither lymph lymphocytes nor macrophages stimulated the autologous PBM cells in culture. Also, the possibility that antigen complexed to products cytophilic for lymphocytes and present in lymph might activate lymph cells could be excluded, after the blood lymphocytes cultured in autologous lymph did not become activated. Activation of lymphocytes during their passage through the cannula by polyethylene does not seem to have been possible since the PBM cells kept in the cannula for 3 h and then for 72 h in culture did not become activated. Since no factors responsible for lymphocyte activation could be demonstrated in lymph, it may be assumed that the process of activation was initiated during the migration of lymph cells through the tissue.

The response curve of lymph cells to stimulation with PHA had a different shape and peak from that of PBM cells. Very low concentra-

tions of PHA evidently stimulated lymph cells, whereas blood cells remained resting. However, the peak response to PHA was in the lymph population lower than in blood. High response to low concentrations of PHA might be due to the additive effects of two signals. One would be the putative tissue "environmental" or "internal" antigens, the other PHA. It is known that the signals initiating mitogenesis are quantitative in nature.

The analysis of the response curve becomes more difficult at high concentrations of PHA when responsiveness of blood cells evidently exceeded that of lymph cells. The large dissimilarity of shapes of both curves suggests that the subset of T-lymphocytes migrating through the skin and recovered in lymph has its functional specificities. This assumption can be further substantiated when the response curves of lymph and blood populations to ConA are compared. Lymph cells responded, at all ConA concentrations, with a much higher rate of [ $^3\text{H}$ ]TdR incorporation than the PBM cells. Usually, a population of immature lymphocytes is more responsive to ConA than to PHA. However, there have been no indications, in our studies, for a local proliferation of lymphocytes in the skin as a potential source of immature forms. The cells responding eagerly to ConA might be the autoreactive T-lymphocytes (14).

The response curves of lymph and PBM cells to PWM did not differ significantly, although lymph cells responded more actively at the lowest concentration. Relatively high, with respect to the lymph cells, response of PBM cells might be due to the more numerous representation of B-cells in blood than in lymph population.

What might be the role played by the skin macrophages in the observed high responsiveness of lymph lymphocytes. It is known that the transfer of the processed and complexed antigen to the lymphocytes may take place by a direct contact with Langerhans cells or indirectly by absorption of the antigen from the tissue fluid (9). *Stingl* et al. (11) have shown that in the skin the Langerhans cells possess the function of macrophages present-

ing antigen to T-lymphocytes. We have observed in lymph lymphocytes being attached to large mononuclear cells, presumably the Langerhans cells, and this strengthens the concept that there may be a cooperation between these two cell types *in vivo*. There were no investigations on the role of Langerhans cells in activation of lymphocytes carried out in the present study. However, our recent unpublished observations indicate that the presence of lymph macrophages in the culture of autologous blood lymphocytes increases their response to PHA by 10 to 12 times.

The high spontaneous transformation rate of lymph cells was found to be inherent to the OKT4<sup>+</sup> enriched population. This high spontaneous activation of helper cells does not seem to be the effect of low activity of lymph suppressor cells. Characterization of lymph cells with the OKT8 monoclonal antibody, specific for suppressor cytotoxic cells, revealed that there was 18% of OKT8<sup>+</sup> cells, compared with 20% in the peripheral blood (7). Also, the responsiveness of the lymph OKT8<sup>+</sup> cells to PHA remained within the same limits as of the OKT4<sup>+</sup> cells.

It could be speculated that the activation of the helper/inducer subset of cells migrating through the tissue is the induction phase of the *in vivo* autologous MLR. Autologous MLR is considered to be an important regulatory mechanism in the immune response with immunologic specificity and memory (14). It has been described that the autoreactive blood T-cells express their own phenotype and specificity. Isolated from the autologous MLR, 90% of them react with OKT4 and 10% with OKT8 antibodies (1, 5, 13).

The responses of the lymph OKT4<sup>+</sup> and OKT8<sup>+</sup> enriched populations to PHA did not differ significantly, although they were evidently higher than in blood. This higher responsiveness of lymph than blood cells may be attributed to the presence in culture of lymph macrophages. The analysis of the differences in response of lymph helper/inducer and suppressor subsets to PHA has been made difficult by the exceptionally high autotransformation rate of both

subsets. The additive effect of the putative activating signal originating from the tissue and of the mitogens made the discrimination of the potency of both stimuli questionable.

In conclusion, the findings of a high spontaneous activation of the prenodal lymph cells which trafficked through the skin and belonging foremostly to the inducer subset, as well as, the evidently higher responsiveness of lymph than PBM cells to mitogens reflect the intensity of immune processes in the normal skin.

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