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SPONTANEOUS CLUSTER FORMATION OF DENDRITIC (VEILED) CELLS AND LYMPHOCYTES FROM SKIN LYMPH OBTAINED FROM DOGS WITH CHRONIC LYMPHEDEMA

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ABSTRACT

To examine the mechanism of spontaneous attachment of afferent lymph lymphocytes to dendritic cells (veiled), we sampled and tested cells from skin lymph in 3 dogs with chronic lymphedema. There were 3.3±2.8% veiled cells in clusters with lymphocytes in lymph obtained directly from dilated dermal lymphatics by percutaneous puncture. The number of ex vivo *clusters forming in the collected lymph samples increased as a function of time and was temperature dependent. The ability of veiled cells to bind lymphocytes was independent of divalent cations but reduced by xylocaine and retinoic acid. Among various steroids tested only methylprednisolone showed an inhibitory effect on cluster formation. Indomethacin and acetylsalicylic acid had no blocking activity on cell binding. Moreover, no effect was seen by cyclosporin A and azathioprine. However, FK 506 had a potent inhibitory effect on spontaneous cluster formation.*

This study suggests that cluster formation by skin lymph veiled cells and lymphocytes is a spontaneous process which can be controlled in vitro.

Skin tissue fluid and lymph contain migrating immune cells, among them large dendritic (veiled) cells (DC) and lymphocytes (1). These cells are continuously transported with the lymph stream to the regional lymph nodes, thereby allowing information about foreign or self-uncovered antigens acquired by these migrating cells in the interstitial space to be transferred rapidly to regional lymph nodes (2). The processing of antigen by veiled cells (VC) and its presentation to lymphocytes seems to start even beforehand in the skin parenchyma. A drop of lymph freshly drawn from a skin afferent lymphatic contains 3-6% of clusters formed by veiled cells and lymphocytes which signifies the *in vivo* cooperation of these subpopulations (3,4). Spontaneous clustering of DC with autologous lymphocytes has been observed *ex vivo* in many species including the peripheral lymph of pigs (5), man (6), and rodents (7).

Clustering is the first phase of antigen presentation to lymphocytes. Dendritic cells are uniquely required for T-dependent immune responses, which occur in cell aggregates or clusters (8). They bind lymphocytes in antigen-independent pathway and also bind resting T lymphocytes (9). Little is known about the mechanism involved in the clustering of DC with resting T lymphocytes including the expression of adhesion molecules. The DC incorporate *in vivo* environmental antigens penetrating the skin or alternatively shed

autologous tissue cell antigens. This response may prompt DC for "spontaneous" clustering with lymphocytes.

In this study, we tried to elucidate the mechanisms of "spontaneous" binding of veiled cells from canine skin afferent lymph with autologous lymphocytes in their natural environment (i.e., in lymph), and in the absence of a known antigen. Control of this process may prove useful in mitigating skin (auto)immune reactions.

MATERIALS AND METHODS

Reagents

The following reagents were used: disodium salt of ethylene diaminetetraacetic acid (EDTA) — Sigma; xylocaine (HCl) (2%) — Astra; heparin — Nova Indust; retinoic acid — Sigma; verapamil (isoptin R) — Yugolek; dexamethasone — Sigma; hydrocortisone — Sigma; methylprednisolone (Solu-Medrol) — Upjohn; cyclosporine A — Sandoz; azathioprine — Wellcome; indomethacin — Sigma; acetylsalicylic acid — Sigma; FK 506 — Fujisawa Phar Co, Lot 029197L.

Dogs

Three outbred dogs with chronic lymphedema after surgical interruption of afferent lymphatics were used as lymph donors (10).

Collection of lymph

Lymph was obtained by direct percutaneous puncture of dilated hindlimb lymphatics and collected into plastic tubes with heparin (10U/ml of lymph). The average concentration of lymph cells was $2.2\pm1.3x10^6$/ml; the percent of cells with VC morphology was 3.2±1.7. Immediately after collecting the lymph, 3.3±2.8% of the whole population of VC were found in clusters with lymphocytes.

Veiled cell-lymphocyte binding assay

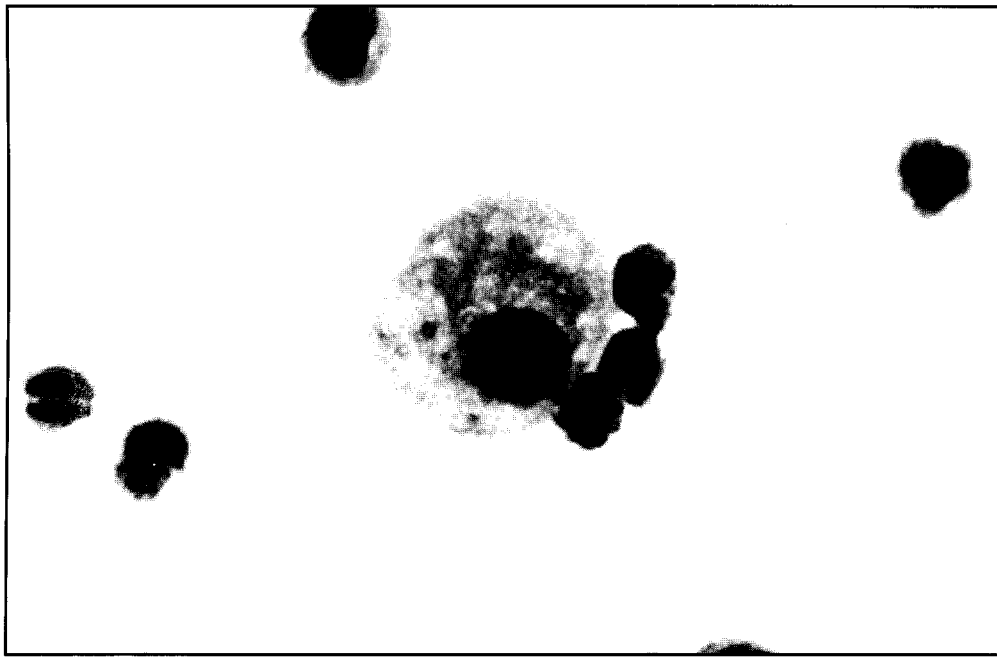

Fig. 1. Cluster isolated from afferent lymph, cytospun onto glass slide and stained with May-Grunwald-Giemsa. 1000x.

VC-lymphocyte binding was quantitated either in lymph immediately after lymph collection or after incubation of lymph cells in lymph mixed 1:1 (v/v) with appropriate reagent solutions. The number of VC with two or more lymphocytes attached *(Fig. 1)* per 100 VC seen in several randomly selected fields was counted by light microscopy (magnification x400). The period of cell incubation with applied agents was chosen depending on the peak of their effect *in vitro.*

Effect of temperature and medium on cell binding

Lymph cells (0.1ml) mixed 1:1 with 0.15M NaCl or autologous lymph were incubated for 4h at 37°C in a tissue culture incubator, at 39°C and 22°C in water baths, and at 4°C in the refrigerator. After incubation cells were gently mixed and clusters were counted.

Treatment with EDTA and verapamil

Lymph cells were mixed with EDTA (10mM and 20mM) and incubated for 1 h, or else with verapamil solution ($5x10^{-3}$M dissolved with 0.15M NaCl, at a concentration of 10^{4} and 10^{5}M) and incubated for 4h at 37°C.

Effect of xylocaine and retinoic acid

Lymph cells were mixed with xylocaine solutions (0.05% and 0.2%) in 0.15M NaCl and incubated for 1 h or else with retinoic acid (dissolved in absolute ethanol to 10^{-2}M and mixed with 0.15M NaCl to a concentration of $5x10^{-3}$M and stored in the dark at 4°C) at a final concentration of 10^{-4} and 10^{-6} and incubated for 4 h at 37°C.

Effect of immunosuppressants and anti-inflammatory drugs

Each steroid immunosuppressant (hydrocortisone, dexamethasone, methyl-prednisolone) was dissolved in ethanol to 10^{-2}M and the stock solution after mixing with 0.15M NaCl was used at the final concentration of 10^{-5}M or 10^{-6}M. Cyclosporine A and azathioprine were dissolved in ethanol to 5 mg/ml as a stock solution and after mixing with 0.15M NaCl were used at the final concentrations of 5μg/ml and 0.5μg/ml. FK 506 was dissolved in ethanol as 10mM stock solution and after mixing with NaCl was used at the final concentrations of 4μg/ml, 0.4μg/ml and 0.04μg/ml. Indomethacin was dissolved in ethanol to 10mg/ml and after mixing with 0.15M NaCl was used at the final concentration of 5μg/ml and 1μg/ml. Acetylsalicylic acid was dissolved in ethanol to 20mg/ml and used at the final concentration of 10μg/ml and 5μg/ml. Lymph cells were incubated for 4 h at 37°C.

To compare results the Student's t-test was used.

RESULTS

Time, medium and temperature dependence of VC-lymphocyte binding

The interaction of lymph cells increased as a function of time during which the cells were incubated together (from 9.5% after 1 h to 20.7% after 4 h incubation; $p<0.05$) *(Table 1)*. After 20 h incubation at 37°C the percentage of clustering was similar (data not shown). *Fig. 2* shows data of cluster formation after 4 h incubation at different temperatures in 0.15M NaCl or lymph. Clustering of cells suspended in NaCl was similar at 37° and 22°C (20.7±2.8% and 16.2±3.3%, respectively). The percentage of clusters formed in the presence of lymph was reduced at temperatures 4°C to 37°C ($p<0.05$), as compared to clustering in NaCl. At 39°C the percentage of clusters both in NaCl and lymph was lower than at 37°C ($p<0.05$).

Treatment with EDTA and verapamil

Treatment of lymph cells with EDTA and verapamil, a Ca^{2+} channel blocker, did not affect the VC-lymphocyte binding rate *(Table 1).*

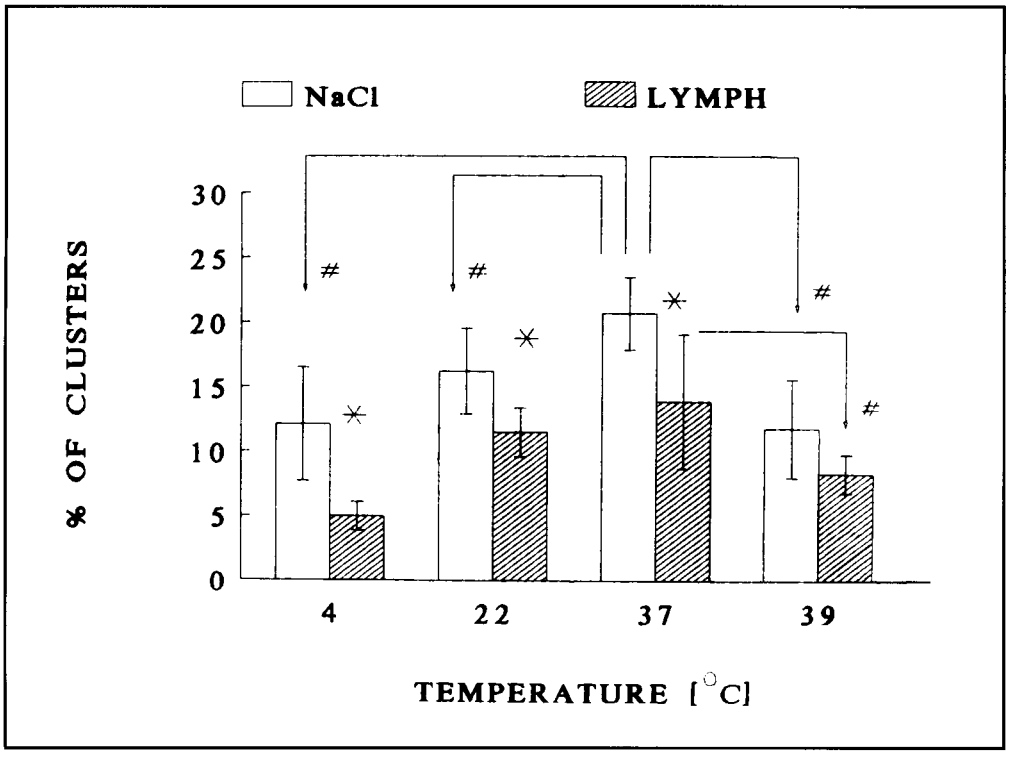

*Fig. 2. Effect of temperature and medium on dendritic or veiled cell (VC) lymphocyte interaction after 4 h incubation. 0.1ml lymph cell samples were suspended in 0.1ml of autologous lymph or 0.15M NaCl. # p<0.05 other temperature vs 37°C; *p<0.05 lymph vs NaCl.*

Effect of retinoic acid and xylocaine

Cell binding was significantly decreased in dose dependent manner when cells were treated with xylocaine and retinoic acid, agents that influence cell membrane fluidity *(Table 1).*

Effect of immunosuppressants and anti-inflammatory drugs

Treatment with methylprednisolone resulted in a decrease in the percent of formed clusters ($p<0.05$) *(Table 2).* Other steroids or immunosuppressants including cyclosporin A and azathioprine and antiinflammatory drugs had no effect on lymphocyte binding. FK 506 had a potent inhibitory effect, even at very low doses.

DISCUSSION

Our studies were undertaken to characterize the spontaneous, physical interactions between circulating skin lymph veiled cells and lymphocytes as observed directly after lymph collection. Understanding of such interactions could broaden insight into veiled cell and lymphocyte cooperation *in vivo* and possibly lead to treatment or prevention of skin (auto)immune response.

Spontaneous binding of VC with lymphocytes proceeded *ex vivo* as a function of time, during which cells were incubated together in the absence of any antigen, reaching a maximal level at 4 h. Cell attachment occurred even at 4°C suggesting the involvement of lymphocytes CD2 and

TABLE 1
Effect of Various Agents on Spontaneous Binding of Lymphocytes by Dendritic (Veiled) Cells of Skin From Peripheral Lymph After Incubation at 37°C

Time of Incubation (hrs)	Cell Treatment (Agent)	(Conc.)	% of Clusters* ($\bar{x} \pm SD$)
0	None	—	3.3±2.8
1	NaCl	0.15M	9.5±6.1**
	EDTA	20mM	16.5±4.9
		10mM	15.0±9.8
	Xylo	0.2%	0.7±0.5†
		0.05%	3.7±1.8
4	NaCl	0.15M	20.7±2.8**
	V-pamil	10^{-4}M	17.0±7.0
		10^{-5}M	23.0±1.4
	RA	10^{-4}M	10.0±2.1†
		10^{-6}M	13.0±1.4†

*Data are the mean±SD of duplicate determinants of three or more experiments.
**p<0.05 4 h vs 0 h and 4 h vs 1 h incubation
†p<0.05 vs NaCl
Xylo—xylocaine
V-pamil—verapamil
RA—retinoic acid
EDTA—disodium salt of ethylene diaminetetraacetic acid
NaCl—sodium chloride

lymphocyte function-associated antigen (LFA)3 adhesion pathway in VC-lymphocyte binding, since LFA1-intercellular adhesion molecule (ICAM) pathway functions only at 37°C (11). Sheep VC express a high level of LFA3 and a slight amount of CD2 (12). Experiments performed at 39°C revealed a reduced cell attachment in the presence of both lymph and NaCl. These findings may be explained by the effect of hyperthermia, as the temperature of canine skin is normally 33-34°C (personal observation), and hyperthermia affects cell adherence in a target-cytotoxic cell system *in vitro* (13). Lower cell binding occurred in the presence of lymph as compared with NaCl, after incubation at 4°C to 37°C. Perhaps some humoral factors present in lymph are involved in these processes and further studies along these lines remain to be carried out.

The present study suggests that the spontaneous binding of lymphocytes to autologous VC is mediated by a divalent cation-independent pathway. Both EDTA and verapamil, a calcium channel blocker did not interfere with spontaneous cell binding. In contrast, active cluster formation

TABLE 2
Effect of Immunosuppressants and Anti-Inflammatory Drugs on Lymph Cell Binding After 4 H Incubation at 37°C

Drugs*	Concentration	% of Clusters ($\bar{x} \pm SD$)
saline (control)	(0.15M)	20.1±3.5
hydrocortisone	10^{-5}M	21.1±7.1
	10^{-6}M	23.5±4.1
dexamethasone	10^{-5}M	18.0±10.7
	10^{-6}M	18.5±3.7
methylprednisolone	10^{-5}M	14.1±5.5**
	10^{-6}M	13.5±4.4**
cyclosporine A	5μg/ml	19.3±5.8
	0.5μg/ml	22.5±5.5
azathioprine	5μg/ml	20.3±5.5
	0.5μg/ml	20.0±4.0
indometracin	5μg/ml	16.3±7.8
	1μg/ml	19.0±7.5
acetylsalicylic acid	10μg/ml	17.8±4.8
	5μg/ml	23.0±6.0
FK 506	4μg/ml	9.1±2.4**
	0.4μg/ml	11.5±2.8**
	0.04μg/ml	12.0±3.2**

*Each drug was dissolved in ethanol, so that the final concentration of solvent was not higher than 0.001%.
**$p<0.05$ vs control (saline)

by canine blood DC with concanavalin A—stimulated lymphocytes in a 3 day culture (14) was blocked by verapamil.

We showed that the ability of VC to bind lymphocytes spontaneously was inhibited by treatment of cells with xylocaine, a drug which interferes with lymphocyte immune functions by affecting cell membrane fluidity, cell adhesion or aggregation (15). Retinoic acid also abolished VC-lymphocyte binding, a response similar to the influence of retinoic acid on efficiency of antigen presentation by spleen DC (16) and lymphocyte response to epidermal cells in a mixed lymphocyte reaction (17).

Glucocorticoids are thought to exert immunosuppressive effects *in vivo* and *in vitro* on both lymphocyte and Langerhans cells from the epidermis (18-20). We attempted to analyze the effect of widely clinically used corticosteroids on cluster formation. Only methylprednisolone used in both physiological and pharmacological doses produced a statistically significant decrease in the percentage of *in vitro* spontaneously formed clusters of VC with autologous lymphocytes. Perhaps other glucocorticosteroids are less potent than methylprednisolone, a drug which affects the generation of cytotoxic lymphocytes *in vitro* by influencing the initial stage of the immune response (21).

The immunosuppressants cyclosporin A (CyA) and azathioprine, which also affect Langerhans cells *in vivo* and their accessory cell function (22) as well as VC functions (23,24), do not prevent spontaneous cluster

formation. These findings are consistent with results of cluster formation by the canine (14) and human (25) blood DC with lymphocytes. However, Furue and Katz (26) note that cultured murine Langerhans cells pulsed with CyA do not form clusters with T cells. These discrepancies may be explained by different experimental protocols. We investigated spontaneously forming clusters of cells freshly obtained from lymphatics and most likely primed *in vivo*, whereas in other reports, cells are cultured and challenged with known antigens *in vitro*.

Whereas the anti-inflammatory drugs, indomethacin and acetylsalicylic acid had no effect on cluster formation, the immunosuppressant FK 506 had a potent inhibitory effect on clustering *in vitro*. Whereas both FK 506 and CyA exert their effect at the same early stage in the lymphocyte activation process, FK 506 binding to lymphocytes during preincubation is stronger and irreversible (27). The potent immunosuppressive effect of FK 506 on prolonging skin allo- and xenograft survival suggests that this agent is superior to CyA and prednisolone (28). Maintenance of local immunosuppression after regionally delivered methylprednisolone in a sponge matrix subcutaneous allograft model (29) raises the possibility of skin immune response prevention at a local level using drugs that affect cell to cell binding.

Although the exact reasons for clustering of lymph cells in the skin tissue fluid *in vivo* remains unclear, it is noteworthy that a large array of environmental antigens (bacterial, viral, fungal) penetrate the skin after microtrauma. Self-antigens of dying tissue cells may also contribute to stimulation of DC. Interestingly, T cells that accumulate in afferent lymph draining the hindlimb of sheep are all of memory phenotype (30).

In conclusion, we suggest that spontaneous binding of VC with autologous lymphocytes in skin lymph contributes to the distinctive capacity of skin Langerhans cells to activate memory/effector T cells *in situ* (31). Controlling this process may be helpful in mitigating skin immune reactivity.

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