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DEVELOPMENT OF ANTISERUM AGAINST VEILED (DENDRITIC) CELLS OF CANINE AFFERENT LYMPH: IMPLICATIONS FOR TRANSPLANTATION BIOLOGY

H. Galkowska, M. Dabrowski, W.L. Olszewski

Department of Surgical Research and Transplantation, Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

ABSTRACT

A method is described for production of anti-veiled cell serum against veiled cells (VC) or dendritic cells obtained from canine skin lymph. By use of discontinuous Percoll gradient, VC from lymph were enriched to about 50% of the entire lymph cell population. After immunization of rabbits with the priming total dose of 10^7 *VC (intramuscular, subcutaneous, intracutaneous) and with the same total booster injection (intravenous), the sera obtained were cytotoxic mainly for VC, with cytotoxin titer 1:16-1:32 and for agglutinin 1:256-1:512, respectively. Antisera used* in vitro *blocked the Ia and CD1 antigens of VC on smears and inhibited the accessory function of VC in cell response to phytohemagglutinin (PHA) and their stimulatory activity in mixed leukocyte reaction (MLR).* In vivo, *the local, intracutaneous administration of antisera led to transient depletion of VC from afferent lymph, and to reduction of mononuclear cells in the T-dependent areas in regional lymph nodes.*

Several authors have suggested that the cells responsible for the initiation of allograft rejection are passenger cells (1). The effectiveness of the Ia-positive veiled passenger cells of the skin collected from afferent lymph in provoking allogeneic response and in initiating renal graft rejection has previously been described (2). A great deal of effort has been expended to eliminate these stimulatory cells from allografts. Nonetheless, treatment with total body irradiation, cyclophosphamide or anti-lymphocyte serum have had only limited success in prolonging allograft survival (3,4). Whole organ pretreatment by simple flushing with anti-class II monoclonal antibodies (moAb) has increased survival of canine renal (5) and rat heart allografts (6). Recently, hemoperfusion with anti-Ia or anti-dendritic cell moAbs prior to intact pancreas allografting (7) and *in vitro* treatment of thyroid allografts (8) has produced increased organ survival. However, the Langerhans cell-dependent antigen presenting function of epidermal cells is unaltered after intraperitoneal administration of anti-Ia moAb, as compared with spleen dendritic cells (9). Whether anti-Ia antiserum reduces the number of Langerhans cells in skin allograft is not known. Also, no data on the possible effects of specific anti-Langerhans cell serum are available. There have been major problems with raising such antisera due to difficulties in obtaining sufficient numbers of dendritic cells from epidermis or afferent lymph. We have found, however, a method for harvesting large numbers of afferent lymph cells, adequate for immunization of antiserum producers.

The purpose of the present study was to

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develop anti-veiled cell antiserum and to characterize its effects *in vitro* and *in vivo* on skin Langerhans cells and lymphocytes.

MATERIALS AND METHODS

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

Collection of Lymph Cells

Lymph (~25-70ml) was collected from dilated skin lymphatics by direct percutaneous puncture. It contained lymphocytes, veiled cells and some granulocytes and macrophages. The total cell number was 2-5x10^7. 7% were veiled cells (migrating Langerhans cells), 4% macrophages, 19% granulocytes and 70% lymphocytes.

Enrichment of Veiled Cells (VC)

Five different concentrations of isotonic Percoll in RPMI 1640 medium with 10% fetal calf serum (Gibco) were prepared (38%, 44%, 50.8%, 55%, 66.7%). After layering of 2ml volumes of Percoll solution, 2ml of a lymph cell suspension in RPMI 1640 medium with 10% FCS was placed on the top and spun down at room temperature at 500xg for 30 min. Low density interface cells were collected and washed with medium. This latter population enriched to about 50% VC also contained lymphocytes. High density interface cells were pure lymphocytes (11).

Immunization Procedure

The priming total dose was 10^7 veiled cells from a low density cell population suspended in 3ml of PBS and then emulsified with an equal volume of complete Freund's adjuvant (Difco). The 2ml portions were injected subcutaneously, intracutaneously and intramuscularly into a rabbit. A total booster injection of 10^7 veiled cells in 3ml of PBS was administered intravenously 3 weeks later (3x in 1ml portions, every other day). Rabbits were sacrificed 7 days after the last injection and sera were collected. They were aliquoted, stored in -20°C and decomplemented before using (56°C for 30 min). A part of sera was absorbed with allogeneic lymphocytes from the canine lymph nodes (2:1) for 2 h at 4°C and next for 30 min at 37°C.

Cytotoxic Titer

Serum cytotoxic titer was checked against the veiled cell enriched population and high density cells. Cells adjusted to the concentration of 2x10^7/ml of RPMI 1640 medium were placed in microculture plate in the volume of 25µl, mixed with 50µl of sera dilutions and incubated at 37°C for 45 min. Then 50µl of diluted 1:15 rabbit complement (Behring) was added and cells were incubated for 45 min. The cell viability was evaluated by 0.2% trypan blue exclusion after mixing 1:1 with the cell suspension. Agglutinin titers were checked by light microscopy simultaneously.

Effect of Antisera on the Expression of Ia and T6 Antigens on Veiled Cells

Cytospins fixed in cold acetone for 1 min were incubated for 30 min with antiserum or with normal rabbit serum as control (both diluted 1:5). Then cytospins were incubated with mouse anti-HLA-DR and anti-T6 (CD1) monoclonal antibodies (Dakopatts, diluted 1:20) for 30 min. Rabbit anti-mouse IgG, alkaline phosphatase-anti alkaline phosphatase (APAAP) complex, alkaline phosphatase (AP)-substrate (Dako) and Mayer's hematoxylin were used for visualizing cross-reactivity with canine DR and CD1 antigens.

Effect of Antisera on the Reactivity of Lymph Cells in Culture

Culture medium consisted of RPMI 1640 medium supplemented with 10mM Hepes buffer, 20% FCS, 100 U/ml penicillin and streptomycin (Gibco) and 2mM L-glutamine (Flow). Whole lymph cells or high

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density cells from Percoll gradient ($2x10^5$) were cultured in 0.2ml medium, both with and without normal rabbit serum or antisera, diluted to a final concentration from 1:8 to 1:96 in the presence or absence of PHA (Wellcome HA15, 90μg/ml). Cultures were performed in triplicate and incubated for 72 h in humidified atmosphere of 5% CO_2 in air. Twenty hours before the culture termination, 100μl of medium was replaced by equal volume of fresh medium in culture and 0.4μCi of ^{3}H-thymidine (Amersham, 2Ci/mM) was added. Radioactivity was measured on glass fiber filters in Permafluor cocktail (Packard). To investigate the direct influence of antisera on the veiled cells only, the low density cells ($2x10^4$) were preincubated with the respective dilutions of antisera or normal rabbit serum for 45 min at 37°C in microculture plate. After washing by plate centrifugation and removing of medium the high density cells were added ($2x10^5$) and cocultured with or without PHA for 72 h. To investigate the effect of antisera on activity of VC in a 6 day mixed leukocyte reaction (MLR), whole lymph cells were cultured with or without 5% VC from the Percoll gradient enriched population. Subsequently cells were preincubated with mitomycin C (Sigma) used at a concentration of 40μg/ml for 40 min at 37°C. Control monoclonal antibodies Dakopatts: anti-HLA-DR (M704), anti-T6 (M721), anti-macrophages (M718) were used in a final concentration of 1:50 and 1:100.

Effect of Antisera on VC Outflow from Afferent Lymph

Lymph was obtained from a cannulated afferent lymph vessel of the hindlimb adjacent to the saphenous vein and collected continuously for 90 min (control) before intracutaneous injection of normal rabbit serum or anti-VC serum (1 ml, diluted 1:1 with 0.9% NaCl), and for the next 150 min after injection, at 30 min intervals. The outflow of lymph cells, including VC, was measured.

Effects of Antisera on Lymph Node

Seventy-two and ninety-six hours after two and three, respectively, intradermal injections in the dorsum of the paw of 1ml anti-veiled cell antiserum or normal rabbit serum (dilutions 1:1), popliteal lymph nodes were harvested and fixed in 4% formalin. Histological sections were stained with hematoxylin-eosin and evaluated microscopically.

TABLE 1
Cytotoxin and Agglutinin Titers of Anti-Veiled Cells (A-VC) Antisera (Before and After Absorption) Compared with Normal Rabbit Serum (NRS)

Cells	NRS	A-VC	Absorbed A-VC
Low density (VC-enriched)			
Cytotoxicity (%)	0	20-40	0-10
Cytotoxin titer	0	1:16-1:32	0-1:8
Agglutinin titer	1:8	1:512	1:64-1:128
High density			
Cytotoxicity (%)	0	10	0
Cytotoxin titer	0	1:16-1:32	0
Agglutinin titer	0	1:256-1:512	0-1:16

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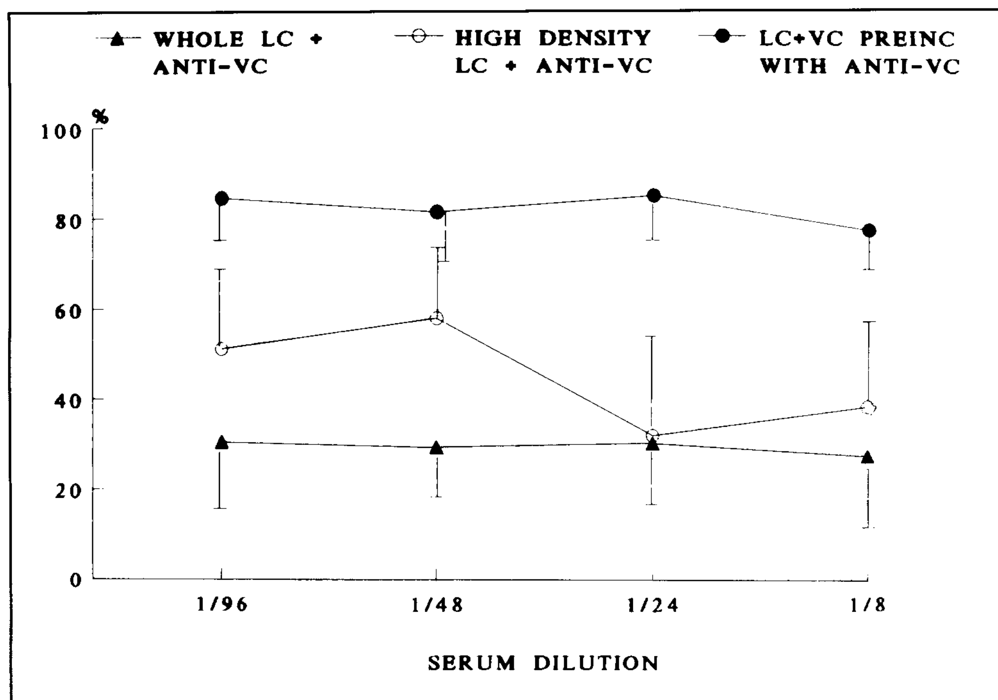

Fig. 1. Effect of anti-VC sera on lymph cell (LC) response to PHA expressed as percent of reactivity in presence of normal rabbit serum in culture. (▲ WLC; (○) HDC; (●) HDC supplemented with 5% VC preincubated with anti-VC serum for 45 min before the onset of the culture. Results are represented as mean percentages ± SD of control cultures, which were supplemented with adequate dilutions of normal sera.

RESULTS

Cytotoxin and Agglutinin Titers

The raised antisera were mainly cytotoxic for veiled cells (20-40% of dead cells), whereas lymph lymphocytes were comparatively resistant (10% of dead cells) *(Table 1)*. Antisera showed a high titer of agglutinins (1:256-1:512) and of cytotoxins (1:16-1:32). Control normal rabbit serum was not cytotoxic and agglutinated only low density cells (titer 1:8). After absorption with allogeneic lymphoid cells, antisera showed decreased agglutinin titers, but were still cytotoxic for veiled cells (0-10% of dead cells).

Expression of Ia and T6 (CD1) Antigens on Veiled Cells

Preincubation of cell smears with antisera before detection of Ia and T6 antigens with monoclonal antibodies led to blocking of these surface antigens, as compared with normal rabbit serum (data not shown).

Lymph Cell Activity in Culture with Antisera and MoAb

Antisera added to the culture inhibited whole lymph cell response to PHA to about 30% of their response in the presence of normal rabbit serum *(Fig. 1)*. When veiled cells were pretreated with antisera before adding lymphocytes to the culture, the inhibitory effect of antisera was still observed (80% of cell response as compared to normal rabbit serum). Stimulatory activity of VC in MLR was blocked by

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TABLE 2
Effect of Anti-Veiled Cells (VC) Sera and Monoclonal Antibodies (moAb) on VC Stimulatory Activity in Mixed Leukocyte Reaction (MLR) (n=3) Data Expressed As Percent of Control Values (Mean±SD)

Agent	Dilution 1:100	1:50
Control serum	115.7±30.0	141.0±21.1
Anti-VC serum	40.3±26.8	66.4±49.2
Anti-Ia moAb	72.4±27.0	65.7±16.8
Anti-T6 moAb	94.4±22.5	109.7±34.3
Anti-MQ* moAb	100.6±21.0	101.4±27.1

*macrophage

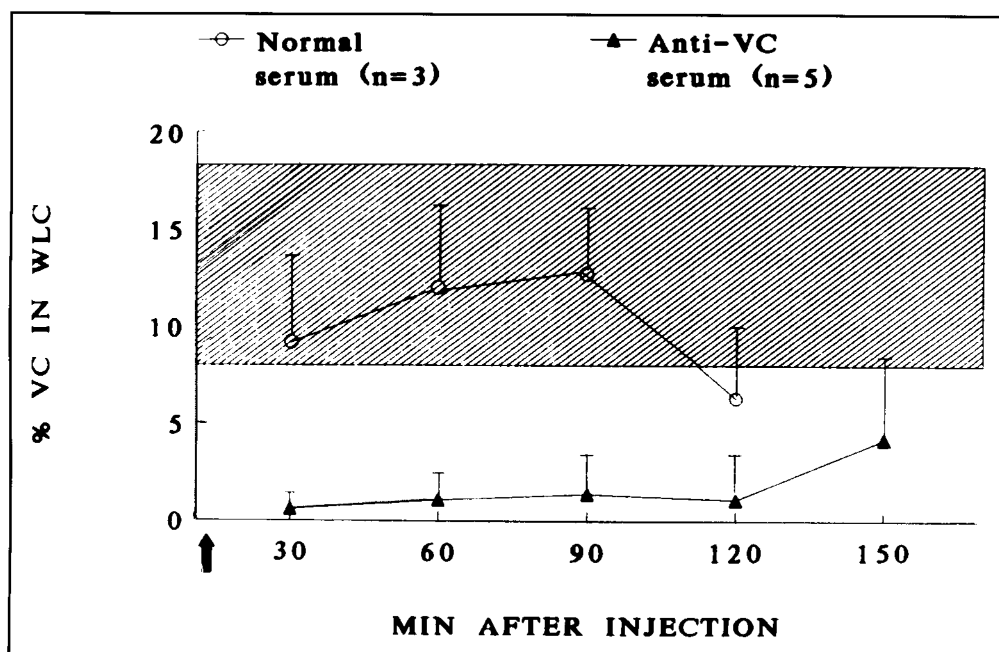

Fig. 2. Effect of anti-VC sera on in vivo *VC outflow from afferent lymph expressed as percent of VC in whole lymph cell population. Collection of lymph samples and cell counts were performed in 30 min intervals before (shaded area) and after the subcutaneous injection of normal serum (○) and anti-VC serum (▲).*

antisera and by anti-Ia moAb as compared with normal rabbit serum and control anti-T6 or anti-macrophage moAbs *(Table 2).*

Effect of Antisera on VC Outflow In Vivo

Antisera were able to reduce the outflow of VC for 150 min after intradermal injection as compared with normal rabbit serum *(Fig. 2)*, leaving the trafficking of lymphocytes thereafter almost intact.

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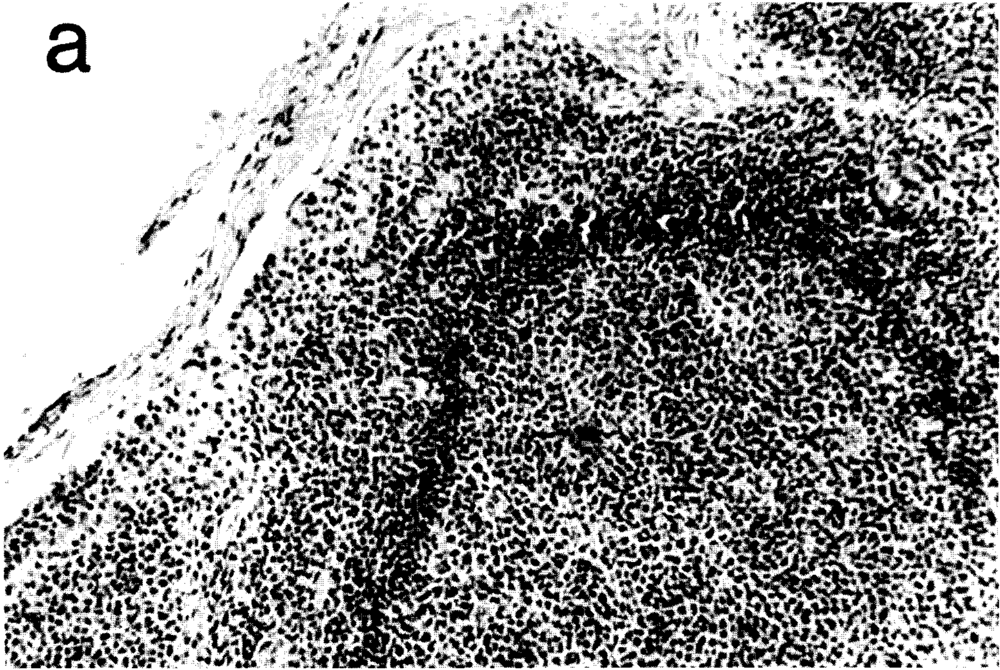
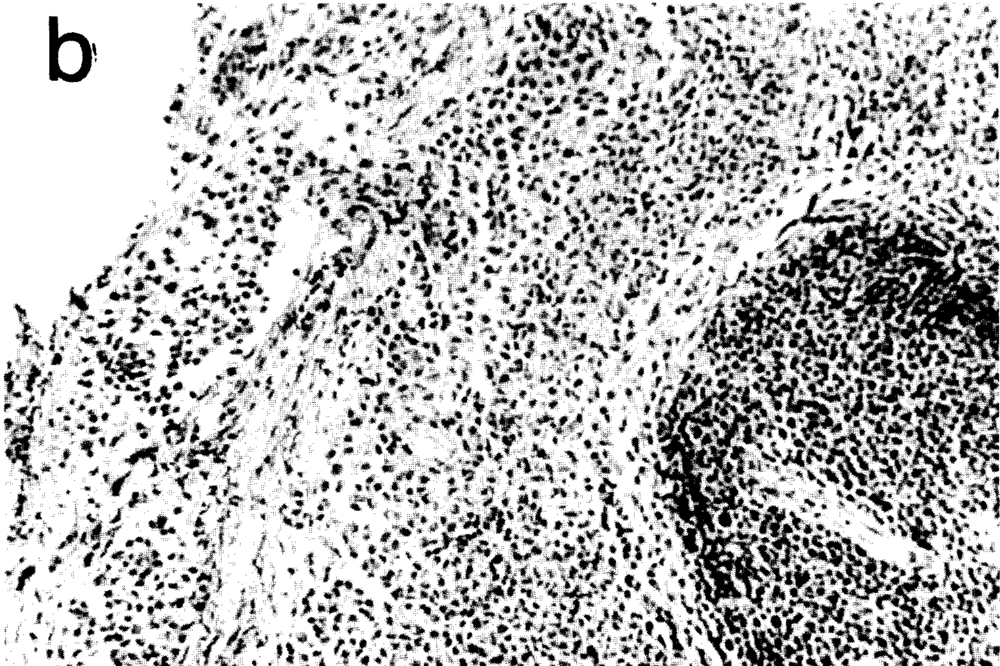
Fig. 3. Photomicrograph of a lymph node after intradermal injection of anti-veiled cell (VC) serum. a: normal rabbit serum; b: two doses of anti-VC serum; c: three doses of anti-VC serum (x250). Note the marked reduction of mononuclear cells in the T-dependent areas with sparing of the follicles after injection of anti-VC sera (b and c).





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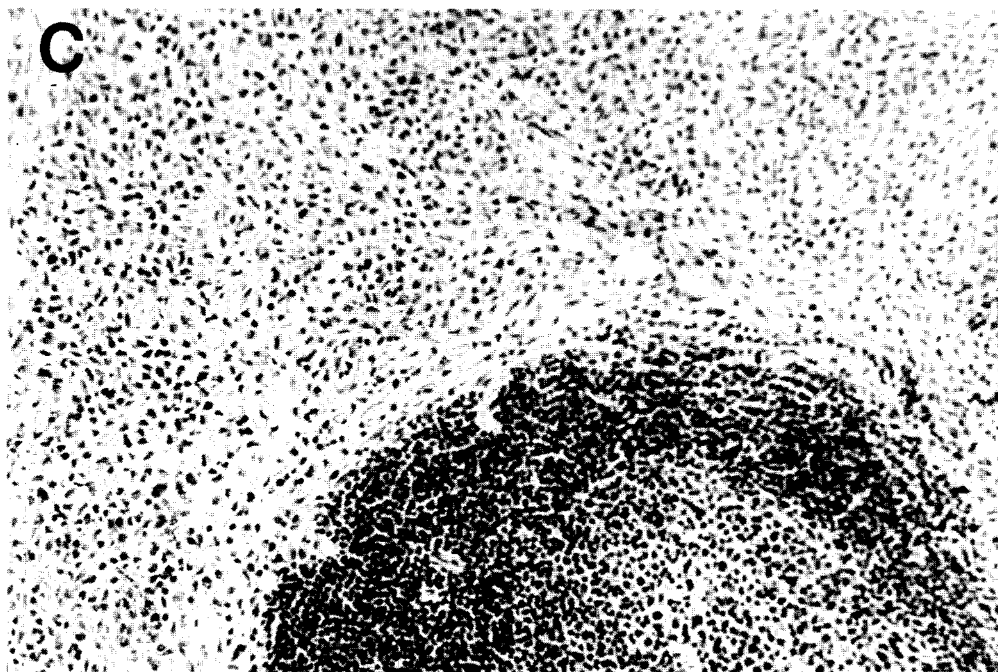

Histological Changes in Regional Lymph Node

Intradermal injection of anti-veiled cell antiserum reduced mononuclear cells in the T-dependent areas and spared follicles in the regional lymph node in a dose dependent fashion. Normal rabbit serum did not produce any changes *(Fig. 3)*.

DISCUSSION

The presence of interstitial dendritic cells in non-hematopoietic tissues has many implications for organ grafting (12,13). Mature dendritic cells constitutively express high density class II antigens and stimulate allogeneic T cells very effectively. Depleting dendritic cells from the transplanted organ would render the host immune system relatively unresponsive to the graft. In skin transplantation, the use of Langerhans cell-free cultured epidermal allografts delays but does not obviate graft rejection (14,15). On the other hand, the presence of Langerhans cells is not sufficient to induce rejection. A necessary step involves induction of Ia on other epidermal cells (keratinocytes) and rejection is blocked by treatment of the host with reagents that interfere with the induction of class II antigens (16).

The rationale for using anti-Ia moAb was to eliminate or prevent Ia-positive cells from interacting with resting T lymphocytes. Perry and Williams (17) demonstrated that survival of skin allografts transplanted across multiple minor or class I, but not class II barriers, was significantly prolonged by anti-Ia moAb treatment of recipients, and was associated with the development of specific suppressor T cells. In the case of dogs, Fuller et al (18) postulated that the presence of canine natural antimouse IgG, can markedly influence the biologic effects of *in vivo* administered moAbs.

In this study, we developed a xenogeneic anti-veiled cell antisera by using veiled cells (VC) from afferent stagnant lymph (lymphedematous dog hindlimbs). Sera were cytotoxic for VC *in vitro*, blocked their Ia and CD1 surface antigens and inhibited VC stimulatory and accessory

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functions in culture. Ladiges et al (19) reported that Ia-like antigens on canine monocytes involved in MLR were cross-reactive with anti-human class II moAbs. We used as a control cross-reactive mouse anti-human moAbs (Dako) against HLA-DR and CD1 antigens. The anti-veiled cell sera developed by us reduced the outflow of VC from afferent lymph after intradermal administration leaving the trafficking of lymphocytes thereafter largely unaffected. Depletion of T-dependent areas in lymph nodes with sparing of follicles with use of the anti-veiled cell sera supports that this antiserum was directed primarily if not exclusively against dendritic cells and T lymphocytes. Moreover, our demonstration that paracortical areas were depleted is consistent with results of Hay et al (20) after local injection of antilymphocyte serum. By arresting the migration of Langerhans cells from graft into lymphoid tissue or their maturation into immunostimulatory veiled cells, a new strategy for overcoming allograft rejection is raised by using antisera against these immunoreactive cells.

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Dr. Hanna Galkowska
Dept. Surg. Res. & Transplant.
Med. Res. Centre
Polish Academy of Sciences
02-004 Warsaw
5 Chalubinskiego Str., POLAND

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