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CELLULAR IMMUNITY TO CANINE MAMMARY TUMOR CELLS DEMONSTRATED BY THE LEUCOCYTE MIGRATION TECHNIQUE

By

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ULVUND, MARTHA J.: *Cellular immunity to canine mammary tumor cells demonstrated by the leucocyte migration technique.* Acta vet. scand. 1975, *16*, 95—114. — Cellular immunity to canine mammary tumor cells was studied by means of the leucocyte migration technique (LMT). Intact tumor cells, separated either by enzymatical or mechanical disruption, were used as antigen, and efforts were made to cultivate tumor cells in vitro. Fifteen female tumorous dogs were studied, and 12 non-tumorous mainly male dags were used as controls. Leucocytes from tumor-bearing females were mixed with own autologous or foreign homologous tumor cells, and control leucocytes were presented with cells from the same source. In addition, leucocytes from tumorous animals and controls were mixed.

Animal group A comprised 8 tumor-bearing females. In this group mixtures of different cell numbers and different tumor cell/leucocyte ratios were tried. Animal group B comprised 7 tumor-bearing females, and 40×10^6 leucocytes from these were mixed with 2×10^6 antigen-cells, antigen-cell/leucocyte ratio 0.05. A great number of tumor cells (tumor cell/leucocyte ratio > 0.05) caused strong non-specific inhibition of leucocyte migration, but in spite of marked inhibition (< 61 %) in the homologous system in animal group A, inhibition in the autologous system was found to be stronger (72.2—92.3 %). In animal group B, dogs presented with own tumor cells showed marked inhibition (23.7—90.1 %), while the controls showed a migration inhibition below 20 %. Mixtures of homologous leucocytes showed inhibition of the same order as mixtures of control leucocytes and tumor cells. Thus evidence of cellular immunity against own canine mammary tumor cells was obtained. It proved difficult to cultivate the tumor cells for more than 2—3 passages. Some evidence of antigenic cross reactivity was obtained between 2 adenocarcinomas. Enzymatical separation of tumor cells did not seem to alter antigenic characteristics of the cell surface. Mechanical separation, however, proved to be simpler, more rapid and yielded cell suspensions largely free of debris, and is therefore recommended for further work.

cellular immunity; tumor antigens; mammary tumors; leucocyte migration technique (LMT); dog.

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Recent reports and experimental data suggest that experimental animal tumors and spontaneous tumors in man are recognized as antigenic by the host. Immune response to tumor antigens involves circulating immune lymphocytes (cellular response) and/or circulating antibodies (humoral response). Sera from tumor-bearing animals and humans often contain antibodies which can block the cytotoxic effect of lymphocytes on target cells in vitro. For review, see *Ulvund* 1972 a, b.

Tumors and immunity have attained only cursory attention in veterinary medicine. Tumor-specific antibody against soluble tumor antigens and cultured canine tumor cells has been found (*McKenna & Prier* 1966, *Powers* 1968, *Yurko et al.* 1969, *Bowles et al.* 1972), and a state of immunologic deficiency has been detected in dogs with mastocytomas (*Howard* 1967). Experiments on tumor vaccination are few, although results have been somewhat promising (*Minton et al.* 1967). Suggestive evidence for the role of cellular immunity in tumor regression (canine oral papillomas) was obtained by *Chambers et al.* (1960).

Mammary tumor virus (MTV) causes mammary cancer in mice, and the tumors contain viral antigens and tumor-specific transplantation antigens (TSTA). Antibodies and cell-bound immunity to MTV-associated antigens have been detected (*Müller et al.* 1971, *Müller & Zotter* 1972), and tumor-bearing animals elicit cellular immunity against individually distinct TSTA, while the animals are tolerant to a common tumor antigen (*Hellstrøm & Hellstrøm* 1969). For review on mammary cancer, see *Ulvund* (1973).

The leucocyte migration technique (LMT) is an in vitro test for cell-mediated immunity. For review, see *Likhite & Sehon* (1971). The technique was adapted to man by *Söborg & Bendixen* (1967) and has lately been described in dogs by *Krohn & Finlayson* (1973).

In tumor immunology, antigens used for the LMT have been soluble tumor antigens (*Bloom et al.* 1969, *Steiner & Watne* 1970) or intact tumor cells (*Malmgren et al.* 1969, *Lespinats & Poupon* 1972). Extracts of autologous mammary carcinomas have been found to induce inhibition of the in vitro leucocyte migration in women with mammary carcinomas (*Andersen et al.* 1969, 1970, *Segall et al.* 1972). Cellular immunity to canine mammary tumor cells has by so far not been investigated. In this work cellular immunity to intact autogenous and homogenous

canine mammary tumor cells has been studied by means of the LMT.

MATERIALS AND METHODS

The LMT was carried out mainly after *Söborg & Bendixen* (1967).

Animals

Different breeds of dogs delivered for surgical removal of mammary tumors at Høvik Animal Hospital, Oslo and at the Department of Obstetrics, Veterinary College of Norway, Oslo, were used. Data on animals used are presented in Table 1. Dogs, mainly males, of different breeds, without any palpable mammary nodules, and delivered at the Department of Surgery for other reasons, were used as controls. Data on these are summarized in Table 2.

Histological procedures

The tumor material was taken immediately after removal of the tumor; 1—2 peaces of each tumor were at once fixed in neutral buffered formalin. These were embedded in paraffin and stained with hematoxylin-eosin and van Gieson by routine technique for histological examination. The tumors included in the investigations were classified according to *Moulton* (1961) and comprised

7 adenocarcinomas (dogs nos. 1, 2, 3, 6, 7, 12 and 13)
6 malignant mixed (dogs nos. 4, 5, 9, 10, 11 and 15)
1 mixed (dog no. 14) and 1 lipoma (dog no. 8). See Table 1.

Tumor cell suspensions

The rest of the tumor was placed in cold Hank's balanced salt solution without antibiotic (*Hoskins* 1967) and shortly after brought to the tissue culture laboratory for preparation of tumor cell suspensions and, if possible, tissue culture.

a) Enzymatical separation: All necrotic, cystic and capsular components were removed. The tissues were minced into small pieces and trypsinized 4 times by routine technique using 0.2 % trypsin at 37°C for 15 min. Finally the cells were resuspended in 2 ml Earles medium (*Hoskins*) and counted (methyl violet staining, methyl violet 50 mg, conc. acetic acid 0.5 g, purified

Table 1. Tumor description in 15 female dogs of different breeds and age.

Case (dog no.)	Breed and age (years)	Tumor size (cm)	Tumor site (n = nipple, l = left, r = right, s = side)	Gross (c = circumscribed)	Histologic diagnosis
1	Poodle, 8	5×4×3	5th n l s	Hard, rugged, poorly c	Adenocarcinoma
2	Eng. set., 11	5×4×4	5th n r s	Soft, poorly c	Adenocarcinoma
3	Cairn terr., 10	5×4×5	„	Hard, fibrous, poorly c	Adenocarcinoma
4	Eng. set., 7½	8×5×5	Betw. 3rd & 4th n l s	Hard with soft areas, poorly c	Malignant mixed
5	Eng. set., 8	2×2×2	5th n r s	Hard, encapsulated, well c	Malignant mixed
6	Eng. set., 11½	5×4×4	„	Hard, fibrous, poorly c	Adenocarcinoma
7	Vorsteh., 7	(2×3×3)×2	4th n r & l s	Hard, rugged encapsulated, well c	Adenocarcinoma
8	Eng. set., 8	3×3×3	Reg. xiphoidei	Soft, round, well c	Lipoma
9	Airedale terr., 9	(3×2×2)×2	4th n r s	Hard, rugged poorly c	Malignant mixed
10	Eng. set., 9	5×3×4	4th n l s	„	Malignant mixed
11	Eng. set., 11	8×5×5	4th n r s	Hard, encapsulated, well c	Malignant mixed
12	Poodle, 6	8×8×8	5th n r s	Soft, round, fairly well c	Adenocarcinoma
13	Cocker span., 11	3×3×4	„	Hard, fibrous, poorly c	Adenocarcinoma
14	Beagle, 6	2×2×2	4th n r s	Rubbery, round, well c	Mixed
15	Eng. set., 12	5×3×2	5th n l s	Hard, fibrous, poorly c	Malignant mixed

Table 2. Anamnestic information in 12 dogs used as controls.

Control no.	Breed, sex and age (years)	Anamnestic inormation
1	Vorsteh. ♂, ½	Cut foot on glass
2	Boxer ♂, 1	Fracture of right radius and ulna
3	Vorsteh. ♂, 1½	Wound, left foreleg
4	Gordonsetter ♀, 10	Phlegmonous inflammation after removal of a toe
5	Collie ♂, 3	Delivered for shortening the teeth
6	German Shepherd ♂, 4	Bilateral hip joint dysplasia
7	Pyrinéhound ♂, 4	Entropion
8	German Shepherd ♂, young	Bilateral hip joint dysplasia
9	Great Dane ♂, 4	Bilateral elbow joint dysplasia
10	German Shepherd ♂, young	Fracture of left femur
11	Irish Wolfhound ♂, 1	Serous inflammation of Bursae olecrani
12	Boxer ♂, ½	Fracture of right femur

water ad 100 g). A fixed number of tumor cells was drawn for use as antigen in the final upset.

b) Mechanical separation: The method was performed as described by *Vaage* (1968) using sterile nylon gauze (Schweizer Nylon Beuteltuch 7/200 μ, Nytal Schweiz). The cells were finally spun down at 1.000 r.p.m. for 8—10 min., resuspended in TC med. 199 (Difco Lab., Detroit, Mich., USA) with 10 % inactivated horse serum, and counted.

Tissue culture procedures

The rest of the tumor cell suspensions (enzymatical separation) was seeded into culture flasks containing Eagle's medium (M.E.M., Wellcome Reagents Beckenham, England). The medium was supplied with 10 % fetal calf serum (FCS, Bio Cult Labs, Glasgow, Scotland), 1.000 i.u./ml of fungizone and 200.000 i.u./ml of polymyxin B. The flasks were incubated at 37°C for 48 hrs. before change of medium. The cells were then allowed to grow for 4—6 days with change of medium every day, and trypsinized when having grown into a monolayer (Antibiotic Trypsin Versene, A.T.V., *Hoskins*). Then the cells were resuspended in Eagle's medium, and cell suspensions for use as antigen were

drawn if required. The rest was seeded into culture flasks. Cells were subcultured through as many passages as possible.

In dog no. 7 cell suspensions from 2 adenocarcinomas, and in dog no. 9 cells from 2 malignant mixed tumors were mixed and used as antigen in the final upsets.

Leucocyte suspensions

Blood was collected from the tumorous dog, just before removal of the tumor, and at about the same time from the controls. Thirty—50 ml of venous blood (anticoagulated with 1 % sodium citrate) was drawn from V. saphena by means of 10 ml sterile evacuated glass tubes (Vacutainer Becton-Dickinson, Rutherford, New Jersey). The blood from each animal was then pooled into an Erlenmeyer flask, and 3 % dextran (mw 5.000.000—40.000.0000, Koch-Light Lab., England) in physiological saline was added to facilitate sedimentation at a volume of 10 ml dextran per 50 ml blood.

After gently turning the flask, the blood/dextran mixture was decanted into 10 ml graduated measuring cylinders and left for sedimentation in a 37°C thermostat for 1½—2 hrs. After sedimentation, the leucocyte-rich plasma was removed as completely as possible, transferred to 10 ml polystyren test tubes and centrifugated at 900 r.p.m. for 10 min. The cells were then washed 3 times in Hank's balanced salt solution previously added 2.5 i.u. of heparin per ml (heparin without preservative, 250 i.u. per ml, derived from Ullevål apotek, Oslo), each time by centrifugating at 900 r.p.m. for 5 min. The samples were mixed in 1 tube to ensure homogenous composition. The cells were then resuspended in 1 ml TC medium 199 containing 10 % inactivated horse serum and counted.

Final mixtures

Group A: Animal group A comprised dogs nos. 1—8 in Table 1 and nos. 1—6 in Table 2. Various cell numbers were used, and tumor cells and leucocytes were mixed in different ratios: 0.8, 0.6, 0.4, 0.1 and 0.05. Equal numbers of leucocytes from 2 dogs (tumorous and control) were drawn and put into polystyren test tubes. They were mixed with a fixed number of tumor cells to give the mentioned cell/leucocyte ratios, in the following way:

Tube 1:	Leuc. x	(Leucocytes from tumorous animal, x)
Tube 2:	Leuc. x + tum. x	(Tum. x = autologous tumor cells)
Tube 3:	Leuc. x + tum. z	(Tum. z = cultured homologous tumor cells from another tumorous dog, (z))
Tube 4:	Leuc. y	(Leucocytes from control, y)
Tube 5:	Leuc. y + tum. x	(Tum. x = tumor cells from tumorous dog, (x))
Tube 6:	Leuc. y + tum. z	(Tum. z = cultured tumor cells from tumorous dog, (z))

The cell suspensions and mixtures were spun down at 900 r.p.m. for 5 min. and finally resuspended in 0.3 ml TC 199 with 10 % inactivated horse serum.

Group B: Animal group B comprised dogs nos. 9—15 in Table 1 and nos. 7—12 in Table 2. Suspensions of leucocytes from tumorous animal (x) and male control (y) were mixed with tumor cells or homologous leucocytes to give a final antigen cell/leucocyte ratio of 0.05 as follows:

Tube 1:	40×10^6	leuc. x		
Tube 2:	„	„ „	$+ 2 \times 10^6$	tum. x
Tube 3:	„	„ „	+ „	tum. z
Tube 4:	„	„ „	+ „	leuc. y
Tube 5:	„	leuc. y		
Tube 6:	„	„ „	$+ 2 \times 10^6$	tum. x
Tube 7:	„	„ „	+ „	tum. z
Tube 8:	„	„ „	+ „	leuc. x

The cell suspensions and mixtures were spun down (900 r.p.m. 5 min.) and resuspended in 0.3 ml TC 199 with 10 % inactivated horse serum.

Incubation procedure

The cell suspensions and mixtures were drawn into capillary tubes (diam. 1.2—1.4 mm, length 7.8 cm). The tubes were sealed at one end by melting, centrifugated at 1.000 r.p.m. for 10 min.

and then cut short below the cell-fluid interface. The cell-containing part of each tube was immediately placed in a plastic culture chamber (plastic-can for Geiger-Müller counting, inner diam. 17 mm, depth 2 mm, derived from Univers mek. verksted, Enskjede, Sweden), and fixed on the bottom and to the edge of the chamber by means of silicone grease (Stopcock grease, non-toxic, Dow corning, USA). The capillaries were mounted in separate chambers, 4—12 parallels being used. The culture chambers were immediately after filled with about 0.5 ml TC medium 199 with 10 % inactivated horse serum, and sealed with cover slips. The chambers were then placed in a 37°C incubator with water saturated atmosphere for 20 hrs.

Sterile procedure was carried out as far as possible. All glassware was sterilized, and plastic culture chambers were irradiated by u.v. light.

Evaluation of the migration

After 20 hrs. of incubation, the round, flat area of migrating cells surrounding the opening of each capillary tube was projected on electrostatic copy paper (L-500, Nashua Corp., Nashua, N.H., USA) by a projection microscope, the outline of each fan was drawn, cut out and weighed. Within 1 set of parallels the variation from one migration area to another did not usually exceed ± 20 %. The migration index (MI) of mixtures of leucocytes and cells used as antigen and of leucocytes only was calculated as follows:

$$MI = \frac{\text{average weight of area with antigen (Mx)}}{\text{average weight of area without antigen (Mo)}}$$

The following formula was used to evaluate the results:
$1 - MI \times 100$ = per cent of migration inhibition with antigen.

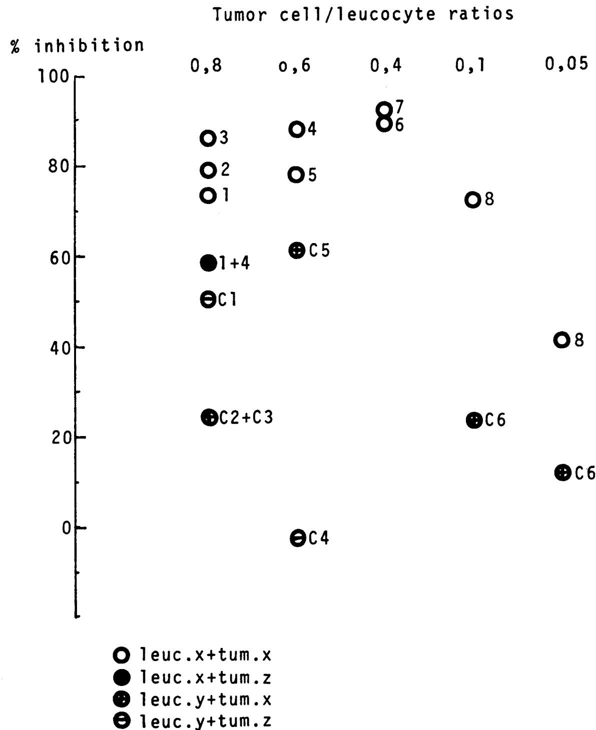
RESULTS

Results of group A (different cell numbers and tumor cell/leucocyte ratios) are presented in Table 3 and Fig. 1. It can be seen that presence of autologous tumor cells (leuc. x + tum. x) caused strong inhibition of migration (72.2—92.3 %) when added in a high number (tumor cell/leucocyte ratio > 0.05). Addition of tumor cells to leucocytes from non-tumorous control dogs (leuc. y + tum. x) also caused strong inhibition of migration,

Table 3. Average weight of migration areas and per cent of migration inhibition, animal group A (different cell numbers and tumor cell/leucocyte ratios were used).

Source of leucocytes (dog no.)	Source of tumor cells used as antigen (dog no.)	Number of leucocytes used (× 10⁶)	Number of cells used as antigen (× 10⁶)	Tumor cell/ leucocyte ratio	Average weight of migration areas (mg)	Migration index (MI)	Migration inhibition (%)
1	—	12.5	—	—	996	—	—
„	1	„	10.0	0.8	267	0.268	73.2
„	4 (1)	„	„	„	411	0.413	58.7
C1 (2)	—	31.25	—	—	682	—	—
„	1 (3)	„	25.0	„	337	0.496	50.4
2	—	25	—	—	2045	—	—
„	2	„	19.0	„	429	0.210	79.0
C2 + C3	—	„	—	—	1085	—	—
„	2	„	„	„	818	0.754	24.6
3	—	16.0	—	—	924	—	—
„	3	„	13.0	„	126	0.137	86.3
4	—	32.8	—	—	428	—	—
„	4	„	19.5	0.6	57	0.118	88.2
C4	—	22.5	—	—	403	—	—
„	4 (4)	„	13.5	„	413	1.025	2.5 (5)
5	—	10.4	—	—	805	—	—
„	5	„	6.25	„	175	0.217	78.3
C5	—	10.6	—	—	392	—	—
„	5	„	„	„	153	0.390	61.0
6	—	12.5	—	—	309	—	—
„	6	„	5.0	0.4	32	0.103	89.7
7	—	45.3	—	—	1475	—	—
„	7	„	18.1	„	113	0.077	92.3
8	—	37.5	—	—	801	—	—
„	8	„	6.25	0.1	223	0.278	72.2
„	„	„	2.0	0.05	468	0.584	41.6
C6	—	„	—	—	637	—	—
„	8	„	6.25	0.1	489	0.768	23.2
„	„	„	2.0	0.05	561	0.881	11.9

(1) First passage (6 days old cultured cells) used.
(2) C = control no.
(3) Monolayer of tumor cells (2 days old) used.
(4) Monolayer of tumor cells (4 days old) used.
(5) 2.5 % increased migration.



F i g u r e 1. Per cent of migration inhibition, animal group A, different cell numbers and tumor cell/leucocyte ratios used. The numbers besides the different markings refer to dog no. or control (C) no.

although the inhibition was somewhat weaker than in the autologous system (< 61 %).

Leucocytes from dog no. 1 (with adenocarcinoma) mixed with cultured malignant mixed tumor cells from dog no. 4 (first passage, cells 6 days old, —leuc. x + tum. z) showed 58.7 % of migration inhibition. The same cultured tumor cells (monolayer from dog no. 4, 4 days old) caused a slightly increased migration in C4 (leuc. y + tum. z). The number of cells and also tumor cell/leucocyte ratio was different, so the results cannot be uncritically compared.

Table 3 shows that leucocytes from different dogs migrated differently in the leucocyte migration system, probably due to genetic heterogeneity, different immunological status, tumor growth or not, and other unknown conditions. In this animal group the variations may also be due to varying leucocyte cell

numbers. Care must therefore be taken in comparing results and drawing conclusions between animals. Because of the few animals tested and heterogeneity of the material, no statistical evaluations on the data have been made.

Because of the slight inhibition in C6 presented with lipoma cells from dog no. 8, ratio 0.05, it was decided to use a fixed number of tumor cells (2×10^6), ratio 0.05, in the following work.

Results of group B (2×10^6) cells used as antigen mixed with 40×10^6 leucocytes, ratio 0.05, are presented in Table 4 and Fig. 2. There was a marked tendency towards strong inhibition in the autologous system (dogs presented with own tumor cells, leuc. x + tum. x) from 23.7 % to 90.1 %. The malignant mixed tumor caused strongest inhibition. One dog with adenocarcinoma (no. 13) presented with third passage of cultured adenocarcinoma cells (13 days old) from dog no. 12 (leuc. x + tum. z) showed 48.6 % inhibition of migration. The mixture of tumor cells from dog no. 13 with own leucocytes (leuc. x + tum. x) was accidentally spoiled. The control dog, C10, presented with the same number and type of cultured adenocarcinoma cells from dog no. 12 showed 6.6 % inhibition of migration.

Leucocytes from control animals were inhibited by homologous tumor cells (leuc. y + tum. x) to a much lesser degree. The very strong inhibition in C8 and dog no. 11 with malignant mixed tumor cells may be explained by the existence of many disrupted tumor cells and lots of cell debris, as marked in the journal. Mixtures of homologous leucocytes, 2×10^6 leuc. y + 40×10^6 leuc. x or 2×10^6 leuc. x + 40×10^6 leuc. y, although with a slight inhibition migrated fairly well together.

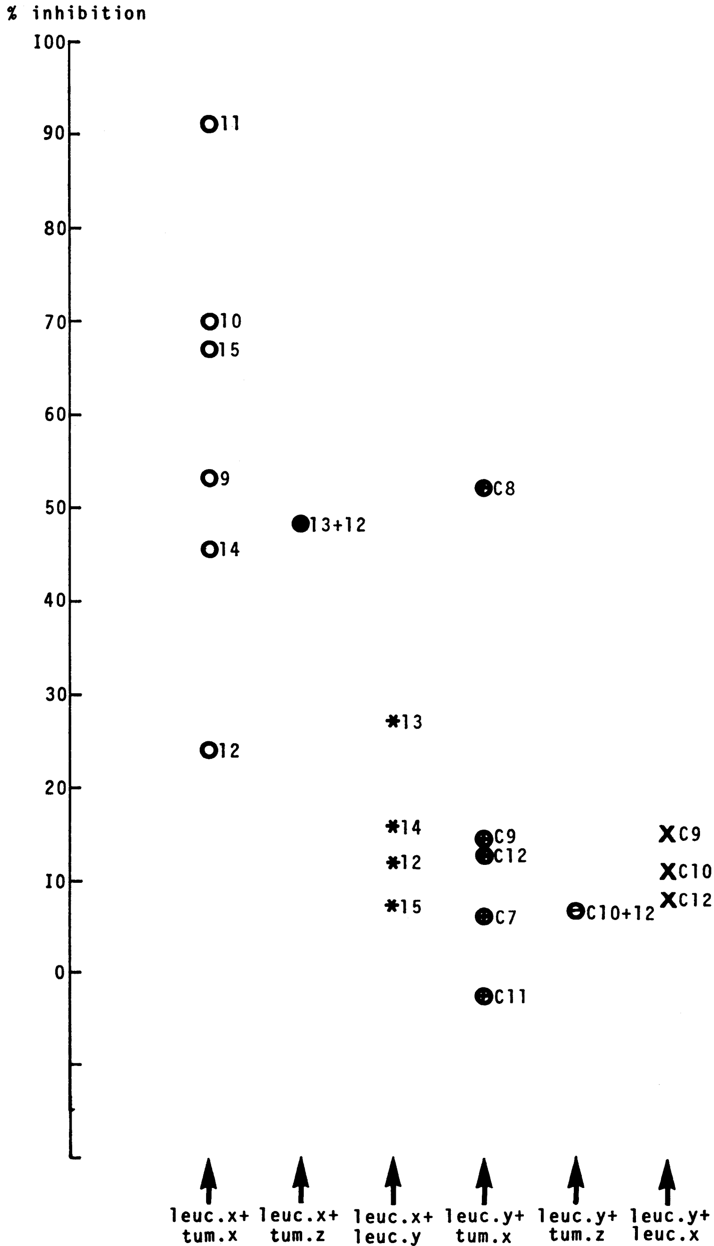
By standard cell culture techniques it proved very difficult to keep the cells alive for more than 2—3 passages — then the growth culminated by slow degrees. One malignant mixed tumor (dog no. 4) was cultivated for 11 passages and kept alive for 75 days.

As will be seen from the tables, some controls and final upsets are lacking. The planned experimental system proved difficult to carry out rigorously because of difficulties in getting tumorous animals at the right time (for use of cultured cells or controls), getting enough cells, and also difficulties, sometimes, in the technical performance of the test.

Table 4. Average weight of migration areas and per cent of migration inhibition, animal group B (2×10^6 cells used as antigen + 40×10^6 leucocytes, ratio 0.05).

Source of leucocytes (dog no.)	Source and type of cells used as antigen (dog no.)	Average weight of migration areas (mg)	Migration index (MI)	Migration inhibition (%)
9	—	1603	—	—
„	tum. 9	749	0.467	53.3
10	—	1756	—	—
„	tum. 10	526	0.300	70.—
C7	—	1455	—	—
„	tum. 10	1368	0.940	6.—
11	—	1080	—	—
„	tum. 11	107	0.099	90.1
C8	—	692	—	—
„	tum. 11	332	0.480	52.—
12	—	300	—	—
„	tum. 12	229	0.763	23.7
„	leuc. C9	264	0.880	12.—
C9	—	442	—	—
„	tum. 12	377	0.853	14.7
„	leuc. 12	375	0.848	15.2
13	—	1110	—	—
„	leuc. C10	809	0.729	27.1
„	tum. 12 (1)	570	0.514	48.6
C10	—	977	—	—
„	leuc. 13	869	0.889	11.1
„	tum. 12 (1)	913	0.934	6.6
14	—	740	—	—
„	tum. 14	403	0.545	45.5
„	leuc. C11	622	0.841	15.9
C11	—	657	—	—
„	tum. 14	675	1.027	2.7 (2)
15	—	1795	—	—
„	tum. 15	590	0.329	67.1
„	leuc. C12	1656	923	7.7
C12	—	1364	—	—
„	tum. 15	1190	0.872	12.8
„	leuc. 15	1245	0.913	8.7

(1) Third passage of tumor cells (13 days old) used.
(2) 2.7 % increased migration.



F i g u r e 2. Per cent of migration inhibition, animal group B, 2×10^6 cells used as antigen mixed with 40×10^6 leucocytes, ratio 0.05. The numbers besides the different markings refer to dog no. or control no.

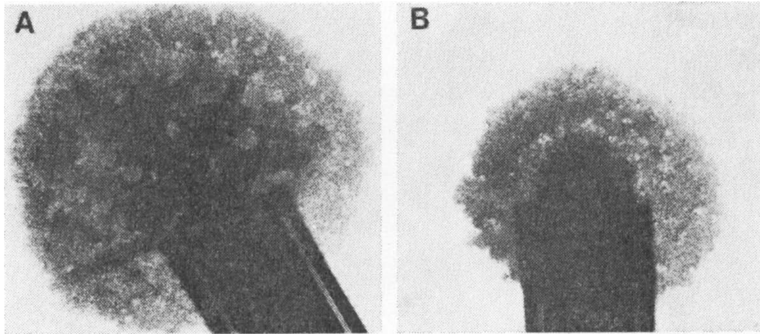


Figure 3. 20-hr. migration cultures. A: migration of 40×10^6 leucocytes. B: migration of 40×10^6 leucocytes mixed with antigen (2×10^6 autologous tumor cells). A marked inhibition of migration can be seen.

DISCUSSION

Because of technical difficulties in getting normal non-neoplastic mammary cells from tumorous dogs, and the possibility of viral and virus induced antigens in normal mammary tissue from tumorous animals (*Hollmann* 1972) leucocytes from tumorous dogs were not mixed with autologous normal mammary cells.

When lymphocytes from 2 genetically different animals are put together in mixed lymphocyte cultures (MLC), the T-lymphocytes will undergo blastic transformation, perform active DNA-synthesis and may divide (*Frøland & Natvig* 1971). In MLC, a mitogenic factor, a cytotoxic factor, a skin reactive factor, a macrophage activating factor and also MIF have been found (*Clausen* 1972). The transformation of lymphocytes reaches its maximum after 3—5 days (*Söborg* 1967, *Malmgren et al.* 1969, *Kalafut et al.* 1972). During the first 24 hrs. the supernatants from MLC have no migration inhibitory effect, but a maximum inhibitory effect is observed in 4 days old cultures. The generation of MIF is probably caused by histoincompatibility between the cell donors (*Clausen*). In cultures of sensitive lymphocytes stimulated by specific antigen, however, MIF has been demonstrated as early as 4—6 hrs. after exposure of sensitized lymphocytes to antigen (*Bloom & Bennett* 1968, *Bendixen et al.* 1972). The early production of MIF in these cultures is probably the result of secondary immune reactions in contrast to the reactions in MLC (*Clausen*). By using genetically different animals as controls therefore, and incubating the cell mixtures for 20 hrs.,

MIF production because of histoincompatibility between the donors should not be expected.

Some concern was given to the utilization of enzymatically separated and cultured tumor cells because of the possibility that enzymatical disruption and culture procedures may alter antigenic and other characteristics of the cell surface (*Carney & Malmgren* 1967, *Vaage* 1968). Several investigators, however, have demonstrated that enzyme treatment used to disaggregate cells does not result in appreciable changes in the antigenic profile of the cell surface membrane, and they have demonstrated tumor specific antigens in established cell lines from a spectrum of tumors (*Hellstrøm & Hellstrøm* 1969, *Di Saia et al.* 1972, *Lespinats & Poupon* 1972). Mechanical disruption of canine mammary tumor cells was in this study performed after *Vaage* in dog no. 14 and dog no. 15, and these results do not differ especially from the rest.

Tissue culture studies were here undertaken in an effort to present leucocytes from tumorous dogs (x) with cultured mammary tumor cells from other tumorous dogs (z) and possibly detect antigenic cross-reactivity between tumor cells. Neoplasma induced by the same virus in different species share cross-reacting tumor-specific intranuclear T-antigens and cell membrane antigens. Chemically and hormonally induced tumors, and tumors induced by irradiation, share no cross-reacting tumor specific antigens. For review, see *Ulvund* 1972 a, b. Slight evidence of antigenic cross-reactivity was obtained between the 3 adenocarcinomas from dog no. 12 and dog no. 13, where the same number and type of tumor cells resulted in 6.6 % inhibition in the control (C10) (Fig. 2).

Varying cell numbers and tumor cell/leucocyte ratios were tried (group A) because of very different ratios reported by other investigators. Equal proportions of tumor cells and macrophages were mixed by *Kronman et al.* (1969). *Lespinats & Poupon* used a tumor cell/macrophage ratio of 1/6, *Cerilli et al.* (1972) 1/21, *Malmgren et al.* 1/80.

The strong non-specific inhibition of migration observed in the controls of group A and especially C8 of group B is probably due to high tumor cell number (group A) and also ruptured cells and cell debris (groups A and B). A great tumor cell number may physically impede macrophage migration (*Malmgren et al.*). *Vaage et al.* (1972) found that the mere presence in the reaction

mixture of killed cells, starch granules or particulate cell debris strongly impeded macrophage motility. *Söborg* found that high concentration of Brucella antigen slightly inhibited the migration of normal cells, but the inhibition was in no case as marked as when the cells came from Brucella-positive individuals.

Erythrocyte sedimentation rate in dogs average 18 mm per hr. and the addition of dextran to facilitate sedimentation (*Söborg & Bendixen* 1967) caused some contamination of red blood cells in the plasma layer. As has been found by others (*Anders & Rindfleisch* 1970), these were not found to impede or affect migration of leucocytes.

CONCLUSIONS

Autologous or homologous tumor cells caused a marked nonspecific inhibition of migration when added in a great number (tumor cell/leucocyte ratio > 0.05) probably because they physically impeded leucocyte migration, and ruptured cells and debris may have been toxic to the leucocytes. Autologous tumor cells caused strongest inhibition, however, while inhibition in the homologous system was somewhat weaker. When 2×10^6 tumor cells were mixed with 40×10^6 leucocytes, ratio 0.05, there was a marked tendency towards strong inhibition in the autologous system, while mixtures of leucocytes from control animals and homologous tumor cells, and mixtures of homologous leucocytes only, showed none or very weak inhibition. Mammary tumors in dogs, therefore, seem to be associated with a state of cellular hypersensitivity against own tumor cells. Both benign and malignant tumors seem to evoke a cellular response. Experiments on blocking antibodies should be included in future. Enzymatical separation of tumor cells did not seem to alter antigenic characteristics of the cell surface. The mechanical technique of *Vaage* (1968), however, proved to be simpler and more rapid, and yielded high numbers of cells in single-cell suspensions largely free of debris. Mechanical disruption of tumor cells is therefore recommended for further studies.

The cultivating of canine mammary tumors by standard techniques proved very difficult, and investigations on antigenic cross-reactivity between different tumors were thus not easy to perform. Slight evidence of antigenic cross-reactivity was obtained between 2 adenocarcinomas. The leucocyte migration

technique is sensitive, equipment needed is simple, the test is relatively rapid, but laborious, and demands strictly defined and standardized conditions with fixed cell numbers and ratios.

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SAMMENDRAG

Påvisning av cellulær immunitet mot jursvulstceller hos hund ved hjælp av leukocytt-migrasjonshemmingstest.

Cellulær immunitet mot jursvulstceller hos hund ble undersøkt ved hjelp av migrasjonshemmingstest (leucocyte migration technique, L.M.T.). Intakte svulstceller, separert enzymatisk eller mekanisk, ble brukt som antigen, og anleggelse av svulstcellekulturer ble forsøkt. Femten tisper med jursvulst ble undersøkt, og 12 dyr uten svulster, hovedsakelig hanhunder ble brukt som kontroller. Leukocytter fra tispe med jursvulst ble blandet med egne (autologe) svulstceller eller fremmede (homologe) dyrkede svulstceller fra en annen tispe. Leukocytter fra kontrollene ble blandet med de samme svulstcellene. I tillegg ble leukocytter fra dyr med og uten svulster blandet.

Gruppe A omfattet 8 tisper med svulst i juret. I denne gruppen ble forskjellige antall celler blandet, og ulike svulstcelle/leukocytt-

forhold utprøvd. Gruppe B omfattet 7 tisper med jursvulst, og 40 $\times$ 10^6 leukocytter fra disse ble blandet med 2 $\times$ 10^6 svulstceller, antigencelle/leukocytt ratio 0,05. Et stort antall svulstceller (svulstcelle/leukocytt ratio > 0,05) forårsaket sterk uspesifikk migrasjonshemming. I gruppe A fant man således en relativt sterk inhibisjon (< 61 %) i det homologe system, men hemmingen i det autologe system ble likevel funnet å være større (72,2—92,3 %). I gruppe B viste tisper presentert med egne svulstceller en hemming på 23,7—90,1 %, mens 5 av 6 hanndyr uten svulster presentert for andre dyrs svulstceller viste en migrasjonshemming som var under 20 %. Blandinger av homologe leukocytter ga hemming av samme grad som blanding av kontrollleukocytter og svulstceller. Det er således sterke holdepunkter for at jursvulster hos hund er assosiert med en cellulær immunreaksjon fra kroppens side.

Det viste seg vanskelig å dyrke svulstcellene lengre enn 2—3 passasjer. Holdepunkter for antigen kryssreaktivitet mellom to adenocarcinomer ble funnet.

Enzymatisk separasjon av svulstcellene så ikke ut til å forandre eller ødelegge de antigene komponenter på svulstcelleoverflaten, men mekanisk separasjon viste seg å være enklere, hurtigere og ga cellesuspensjoner som for størsteparten var fri for ødelagte celler og cellegrums. Mekanisk separasjon anbefales derfor ved videre undersøkelser.

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