HYALURONIDASE-SENSITIVE HALOS AROUND ADHERENT CELLS

Their Role in Blocking Lymphocyte-Mediated Cytolysis

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Tumor cells can protect themselves in vitro and in vivo against immune assault by such mechanisms as the secretion of nonspecific suppressive or anti-inflammatory factors (1) or the shedding of tumor-specific antigens (2). In this report we describe a further mechanism which can prevent immune effector cells from establishing contact with tumor cells in vitro and which, in so doing, inhibits lymphocyte-mediated cytolysis.

During attempts to perform cell-mediated cytotoxic assays using adherent fibrosarcoma cells as targets, tumor cells were seen to be surrounded by a large transparent halo which spleen cells were unable to penetrate (Figs. 1 a and 2 a). This report deals with the nature of this barrier between tumor and effector cells. It shows how it can be removed and how its removal allows lymphocyte-mediated cytolysis, a reaction inhibited by its presence. Such barriers have been demonstrated on synovial and fibroblast cells (3, 4, 5); however, their presence on tumor cells and their role in preventing cell-mediated cytolysis of such cells does not seem to have been previously reported.

Materials and Methods

Cells. The tumor cells most intensively investigated were derived from a fibrosarcoma, induced in C3Hf/Bu mice by methylcholanthrene treatment. Serially transplanted flank tumors in the 7-12th generation were taken and cell suspensions prepared as described previously (6). Cells cultured in vitro for up to 15 passages were used in the experiments. A range of other cells were also examined: fibrosarcoma cells W1, W5, W9, W14, W111, W112, and W114 were kindly provided by Professor M. F. A. Woodruff and Mrs. Gillian Speedy, lymphoblastoid cells by Dr. M. Steele, 3T3 cells by Mr. W. Christie, Medical Research Council Cytogenetics Unit; mammary carcinoma cells SP22 and 40A by Dr. M. Moore, Christie Hospital and Holt Radium Institute, Manchester; VERO and BHK-21 cells by Mr. D. Brown, Bacteriology Department; the other tumors used were maintained in our laboratory; fibroblasts from mouse 14-d-old embryos and adult human skin were also used. Additional information on the cells is provided in the text. In all experiments, cell cultures were seeded sparsely at densities of 10^4 cells cm^{-2} on coverslips or in 96 well Microtitre plates No. M29 ART (Sterilin, Teddington, England). Eagle's medium (Wellcome, England) supplemented with 10% fetal calf serum (Gibco, Bio-cult, Paisley, Scotland) and buffered with 20 mM Hepes (British Drug House Chemicals, Poole, Dorset) was used throughout. The formation of halos was dependent on the presence of the serum.

Cell suspensions were prepared from C3Hf/Bu mouse spleens and washed once before use. A T-lymphocyte-enriched population of spleen cells, for use in the microcytotoxicity tests, was separated from other spleen cells by nonadherence to nylon wool (7). These suspensions contained 80–85% T cells (8).

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The optimal ratio of spleen cells to tumor cells for visualising the halos was $\cong 3.0 \times 10^6$ cm^{-2}, i.e., just sufficient to form a cell sheet. 4×10^9 cm^{-2} formalin-fixed *Corynebacterium parvum* organisms were also used to visualise the halos as was Pelikan, shellac-free, drawing ink (Gunther Wagner, Hanover, Germany). It was found that the process of visualisation could be hastened by spinning the particles onto the cells using a conventional centrifuge with plate holder or a cytocentrifuge for the slide/coverslip preparations.

Microscopy. Whereas the halo phenomenon could readily be seen using a conventional inverted microscopy, it could be studied in greater detail if cells were plated on to circular coverslips and placed in a Dvorak-Stotler chamber (9; Zeiss, Ober Kochen, W. Germany) or if the coverslips were allowed to hang from the underside of a thin slide. Here they were examined and photographed under Nomarski optics on a Zeiss Universal microscope. For time-lapse studies, cells were plated on Falcon plastic dishes with Cooper lids (Becton Dickinson UK, Ltd., Middlesex) and filmed on a Wild inverted phase microscope (Wild, Heerbrugg, Switzerland) with Wild time-lapse equipment in a 37°C room (10). Lapse rates of 10–100 s were used.

Enzymes. Hyaluronidase from bovine testis type 1 (Sigma, Kingston-on-Thames, England) and ovine testis (Sigma), both of which also degrade chondroitin sulphate, and hyaluronidase *ex Streptomyces* (11) grade B (Calbiochem, San Diego, Calif.) which is specific for hyaluronic acid, were used in the study. These enzymes had no detectable activity against the general proteolytic substrate azocoll (Calbiochem) and the activity of the fungal hyaluronidase was resistant to heating at 60°C for 30 min (11). In other attempts to remove the barriers, cells were treated with concentrations of trypsin, pepsin, and pronase (BDH) varying from 0.02 to 0.0002% for 20 min at 37°C; 0.002% DNase I (Sigma), 15 min at 37°C; 0.02% RNase (Sigma), 15 min at 37°C; and 50 U/ml *Vibrio cholerae* neuraminidase (BDH), 15 min at 37°C. Tests were performed in Eagle's medium containing 10% FCS and in Dulbecco's phosphate-buffered saline (PBS); the enzymes were removed by washing and the cells were tested immediately for their ability to form halos using spleen cells as indicators.

Microcytotoxicity Tests. 10^4 tumor cells were plated in each well of a 96 well Microtitre plate No. M29 ART (Sterilin). After overnight incubation, 10 μCi $Na^{51}CrO_4$ (Radiochemicals Centre, Amersham, Bucks.) were added in 50 μl medium. After 2 h at 37°C, the wells were washed five times with warm Dulbecco's PBS and the appropriate number of spleen cells added to each well. After a further 7-h incubation period, the supernate was removed, the cells washed once in Dulbecco's PBS, and the label associated with the cells was released by treatment with 0.1 ml 2 N NaOH. The counts in the experimental wells were subtracted from those in control wells to which no spleen cells had been added; this value was then expressed as a percentage of the control counts to give the cytotoxic index. When required, cells were treated with 10 IU/ml (0.1-ml final volume) bovine testicular or fungal hyaluronidase during the labeling and also during the subsequent incubation periods.

Results

Our initial observations were made with C3Hf/Bu fibrosarcoma (Fsa) cells. Typically, after culture for longer than 2–4 h, ≅80% of these cells prevented normal spleen cells from coming close to their cell membranes. The effect was made obvious by the presence of large translucent halos around the tumor cells which spleen cells were unable to penetrate (Figs. 1 a & 2 a). No structure was visible in the halo region by either phase contrast, dark ground or Nomarski optics; the halos extended well beyond the filopodial attachments seen with high resolution microscopy (Fig. 1 a).

In one experiment halo sizes were measured; they ranged up to 17 μm, averaging 8.8 μm, from Fsa membrane to spleen cells; the minimum measurable size was 2 μm. Halos were formed by cells taken in vitro directly from a tumor or by cells subcultured for up to at least 15 passages in vitro.

Time-lapse cinemicrography showed that at no time did spleen cells penetrate the halos; they were excluded even as they settled on to the surface of the dish, the settling pattern suggesting that the halos were not simply associated with the substratum but

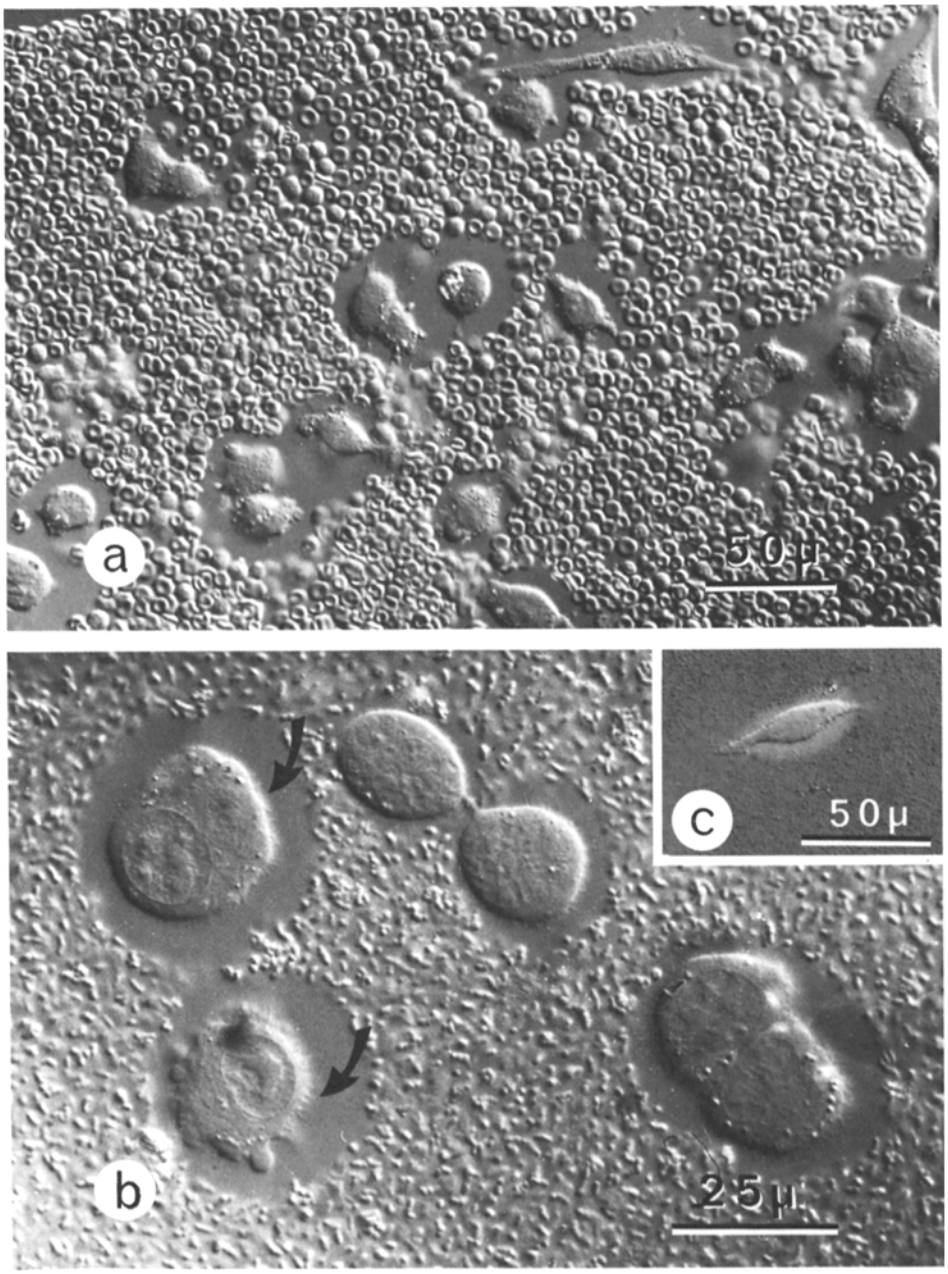
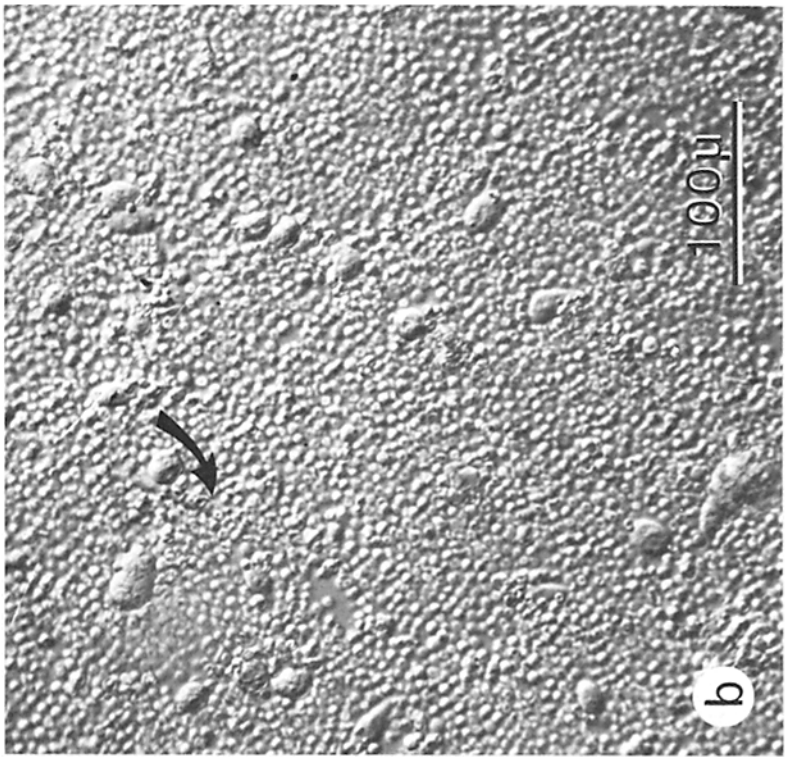


FIG. 1. Living Fsa cells photographed under Nomarski optics showing the halo phenomenon in the presence of (a) spleen cells, 350 ×; (b) fixed *C. parvum* organisms, × 850; (c) carbon particles, × 350. Note that under high magnification (b) the halos extend beyond the fine processes coming from the cell body (arrows). Cells were plated 16 h before the indicator particles were added and photographed 10 min later.

extended above and around the Fsa cell. Motile cells had very prominent barriers around their trailing edge but the halo tended to be much less pronounced at their leading edge (Fig. 2a, see legend for details).



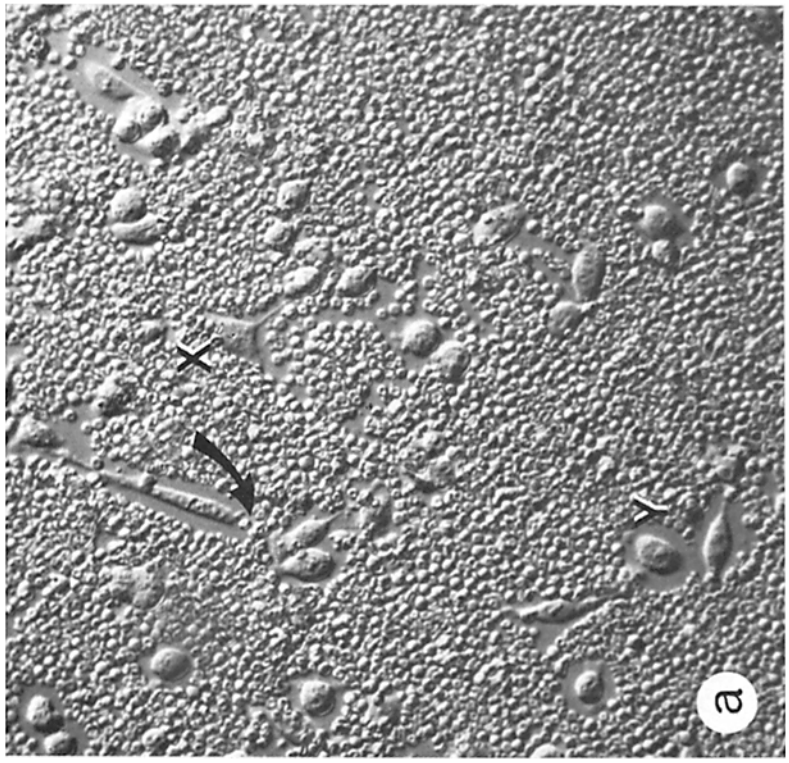


FIG. 2. A field of Fsa cells and mouse spleen cells prepared as for Fig. 1 a and photographed before (a) and 10 m after (b) the addition of *Streptomyces* hyaluronidase (final concentration 2.5 U/ml). Note that in (a) most of the Fsa cells are surrounded by a halo that spleen cells are unable to penetrate. Certain cells (arrow x) have less halo on one side than the other. Time-lapse cinematography suggested that such cells were motile and that the barrier was much less pronounced on the leading edge. Certain rounded cells (y) had very prominent halos. In (b), hyaluronidase treatment has removed the halos (compare the elongated cell marked with an arrow in the two micrographs) and many of the Fsa cells appear to be covered with spleen cells × 210.

The exclusion phenomenon was seen with particles other than normal spleen cells. Bacteria (Fig. 1 b) and carbon particles (Fig. 1 c) showed halo formation as did immune spleen cells, lymph node cells, thymocytes, normal peritoneal exudate cells, and erythrocytes. Visualisation of the halos was optimal when the concentration of indicator particles was sufficient to form a single layer sheet: at lower concentrations, the monolayer was insufficient to see halos, whereas at high concentrations, spleen cells piled up over both the Fsa cells and their halos. It was of interest that fixation of the tumor cells in 1% formal saline or 2.5% glutaraldehyde did not remove the barrier; after dehydration, however, the halos were no longer visible.

The C3Hf/Bu Fsa cells were not alone in their ability to exclude particles from the region around them. A variety of normal and tumor cells also were found to possess halos (Table 1). A major exception, however, was the seven lymphoblastoid cell lines tested. Vero cells also appeared to be negative. It should, however, be noted that halos of <2 μm would not be demonstrable.

These barriers are similar to the hyaluronidase-sensitive material seen to surround synovial cells and fibroblasts in vitro (3, 4, 5). We therefore examined the sensitivity of the halos formed by the cells detailed in Table 1 to bovine testicular hyaluronidase (10 IU/ml). Treatment with hyaluronidase removed the barrier so that the spleen cells were then able to approach the cell membranes (Fig. 2); with most cell types, the barrier was removed within 3 m, although certain of the carcinoma cells appeared to be slightly less sensitive. These concentrations of hyaluronidase appeared to have no adverse influence on the morphology or growth of the cells. After removal of the enzyme, the cells regenerated their ability to repel spleen cells within 2 h. Fungal (0.1–10 IU/ml) and ovine testicular (10 IU/ml) hyaluronidase where tested, had the same effect. Other enzymes (pronase, pepsin, trypsin, neuraminidase, collagenase, DNase, and RNase) did not remove the barrier when used at concentrations that did not obviously affect the morphology and adherence of the cells to glass.

These results show that many adherent sarcoma and carcinoma cells, as well as normal cells, are surrounded in vitro by hyaluronidase-sensitive zones that can prevent spleen cells as well as other cells and particles approaching their cell membranes. As it is thought that contact between effector and target cells is a prerequisite for cell-mediated cytolysis (12), we have investigated the extent to which these halos could protect tumor cells from lymphocyte-mediated lysis.

For these experiments, cells were prepared from the spleens of C3Hf/Bu mice that had been bearing Fsa tumors for 14–21 d. These spleen cells are known to possess anti tumor activity as measured in a Winn assay (8). The ability of the cells to lyse Fsa cells was measured in the presence and absence of hyaluronidase by a ^{51}Cr-release assay. The results of four separate experiments are presented in Table II. Hyaluronidase did not effect $Na^{51}CrO_4$ uptake by tumor cells; it did not significantly increase the ^{51}Cr released by tumor cells cultured with normal spleen cells. It did, however, in all experiments, enhance the ^{51}Cr released by Fsa cells in the presence of specifically immune spleen cells; the average increase in lysis being around threefold. The enhanced killing of Fsa cells by immune spleen cells in the presence of hyaluronidase was most obvious when nylon wool nonadherent (T-cell enriched) spleen cell populations were used (exps. 3 b and 4 b; Table II); the major reason being that T-cell-enriched preparations displayed less nonspecific cytotoxicity than did the unseparated spleen cells. The cytotoxic action of immune spleen cells in the presence of hyaluronidase was immunologically specific in that, in the four experiments, we found no

TABLE 1

Cell type	Comment	Strain/species	Coat
Fsa 1	MCA*-induced	C3Hf/Bu mice	+ve
Fsa W1, W5, W9, W14	MCA*-induced	CBA mice	all +ve
Fsa W111, W112, W114	MCA*-induced	Balb/c mice	all +ve
Mammary carcinoma 2	Spontaneous	C3Hf/Bu mice	+ve
Mammary carcinoma SP22	Spontaneous	W/not rats	+ve
Mammary carcinoma 40A	AAF‡-induced	W/not rats	+ve
Adenocarcinomas 1, IM, 2	Spontaneous	C3Hf/Bu mice	all +ve
3T3 G	Fibroblast-like	Swiss albino mice	+ve
3T3 S	SV_{40} transformed	Swiss albino mice	+ve
BHK-21	Fibroblast-like	Hamster	+ve
Mouse embryo fibroblasts		C3Hf/Bu mice	+ve
Adult skin fibroblasts		Human	+ve
Vero	Fibroblast-like	Monkey	−ve
B-lymphocyte lines EB_4, CRO_1, ANA_1, $G\text{-}S_1$, JIM_1		Human	all −ve
Myeloma 8226		Human	−ve
T-lymphocyte line $MOLT_4$		Human	−ve

* methylcholanthrene-induced
‡ N acetyl-α-amino-fluerene-induced

enhanced cytotoxicity against C3Hf/Bu adenocarcinoma IM cells when the enzyme was added (data not shown). These experiments show that the hyaluronidase-sensitive zones around tumor cells, in preventing immune lymphocytes from approaching their cell membranes, can protect them from specific cell-mediated cytolysis in vitro.

Discussion

The results show that, when cultured in vitro, cells from a C3Hf/Bu fibrosarcoma are surrounded by zones that particles cannot penetrate and that these zones, which are removed by hyaluronidase, can protect the cells from lysis by immune lymphocytes. A variety of other sarcoma and carcinoma cells as well as normal cells had similar exclusion zones; the lymphoblastoid cell lines that we tested did not.

There are only a few reports in the literature of similar phenomena, a fact that is perhaps surprising. Halos of a similar thickness have been noted on human synovial cells (3, 4) and their presence considered sufficient to protect such cells against leukocyte-induced damage (5) and to inhibit viral adherence (13). A similar reaction with erythrocytes has been attributed to human polymorphonuclear leukocytes (14) but the hyaluronidase-sensitivity of the halo was not investigated in that paper. We have been unable to find reports of exclusion zones around tumor cells. We believe the reason for the lack of such reports is that they are difficult to visualise. They appear only 2 h after the cells are in culture and their identification requires indicator particles to be present at the correct concentration.

The nature of the halo is largely unknown. The evidence that we have is most compatible with its being a gel-like coat extending beyond the filopodia, encompassing the cell mass and being composed in part of hyaluronic acid. Very low concentrations of this glycosaminoglycan, perhaps complexed with protein and other polysaccharides (15) could form coats with properties consistent with those we have described as it is known that hyaluronic acid is heavily hydrated (16). Support for this view comes

TABLE II

Effector cells	Hyaluronidase*	Lysis‡							
		Immune§				Normal			
		125:1‖	Incr.¶	50:1	Incr.	125:1	Incr.	50:1	Incr.
		%							
Exp. 1									
spleen cells	−	14	3.5[a]	9¶	>1.9[a]	14	1.3	11	1.5
	+	49		19		17		16	
Exp. 2									
spleen cells	−	24	1.4[a]	12	2.0	16	1.5	21	1.3
	+	34		24		24		28	
Exp. 3 a									
spleen cells	−	10	2.8[a]	2¶	>2.8[a]	18	<1.0	17	<1.0
	+	28		28		10		10	
Exp. 3 b									
T-cell-enriched** spleen cells	−	0¶	>2.7[a]	9¶	>3.2[a]	0	1.0	3	1.0
	+	27		32		5		0	
Exp. 4 a									
spleen cells	−	14	3.8[a]	29	1.1	51‡‡	1.2	39	1.4
	+	53		33		61		54	
Exp. 4 b									
T-cell-enriched** spleen cells	−	13	2.9[a]	0¶	>4.1[a]	17	1.0	1	1.0
	+	38		41		16		9	

* 10 IU/ml (0.1-ml vol) bovine testicular (exp. 1, 2, and 4) or fungal (exp. 3) hyaluronidase present.

‡ 1 − (counts remaining in wells with added spleen cells + counts remaining in wells with no spleen cells) × 100%. This method of calculating precent lysis gave more reproducible results than supernatant counts although they could equally well have been used. Nonspecific release ≅30%. Four replicates were averaged for each percent lysis.

§ Suspensions from spleens of mice bearing Fsa for 14–21 d (≅12 mm diameter tumors).

‖ Effector:target cell ratios.

¶ Lysis in the presence of hyaluronidase divided by lysis in the absence of hyaluronidase. Statistically significant increases ($P < 0.05$; Student's t test) are shown by [a]. Where lysis was <10%, then 10% was used for the calculation of the increase.

** T-cell enriched = nylon wool nonadherent.

‡‡ Very occasionally high cytotoxicity was found with supposedly normal spleen cells. The explanation for this is presently unknown.

from the fact that dehydration (though not fixation) removes the halos. Alternative hypotheses, for example that the barrier is due to fine, active cell processes, require to explain the exquisite sensitivity of the halos to the several different types of hyaluronidase and in particular to the highly specific, heat stable fungal hyaluronidase.

The importance of these halos to the cells is hard to define. They may act as a protective mechanism against the harsh conditions in vitro, yet the cells appear not to be affected by removal of the halos with hyaluronidase. In the experimental context, we have shown that halos prevent the approach of immune effector lymphocytes to tumor cells and, in so doing, appear to protect the tumor cells against lysis. The phenomenon may therefore influence the outcome of certain microcytotoxicity assays. It is known that there are particular problems in performing ^{51}Cr-release microcytotoxicity assays using adherent cells as targets; for such assays, lymphoblastoid cell lines are most commonly used (17). In this regard it may be significant that

we could not detect halos around any of the lymphoblastoid cell lines tested.

It is clear that the addition of hyaluronidase may under certain conditions facilitate the cytotoxic performance of immune cells against adherent cells in vitro. However, other related factors may be important in the performance of these assays; these include the extent of halo production and its chemical composition, the time at which effector cells are added with respect to the time taken for the establishment of exclusion zones by the targets, and the presence or absence of cells such as activated macrophages which may have enzymes capable of breaking down the halo.

The significance of halo formation by cells in vitro to their behavior in vivo is as yet unclear. It is however of interest that hyaluronic acid is a major component of the ground substance and as such it will be present within solid tumors and would be expected to help maintain the integrity of the tumor. Although it has been suggested that breakdown of ground substance may facilitate spread of the tumor (18), the results presented here suggest that it would be worthwhile investigating whether its removal may in fact facilitate penetration and elimination of the tumor by the host's immune defences.

Summary

A variety of adherent sarcoma, carcinoma and normal cells are surrounded in vitro by thick, transparent zones (≅9 μm thick) that spleen cells and a variety of other cells and particles cannot penetrate. Seven lymphoblastoid cell lines did not possess such halos.

The presence of these halos around adherent fibrosarcoma cells appeared to protect them from lymphocyte-mediated cytolysis. Hyaluronidase treatment, which destroyed the halo and allowed lymphocytes to approach the tumor cell membrane, enhanced the cytotoxic action of immune but not of normal spleen cells.

These observations, in addition to highlighting a little-known feature of the cell surface, may also be of general relevance to the in vitro and in vivo killing of tumor cells by immune effector cells.

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