Interaction of Choriocarcinoma Cells and Human Peripheral Blood Lymphocytes

RESISTANCE OF CULTURED CHORIOCARCINOMA CELLS TO CELL-MEDIATED CYTOTOXICITY BY MITOGEN-ACTIVATED LYMPHOCYTES

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ABSTRACT Cultured choriocarcinoma (BeWo) cells exist that share many of the morphologic and biosynthetic properties of normal human trophoblasts. In an attempt to develop a model for the immunologic relationship between a sensitized mother and fetus, we mixed BeWo cells with mitogen-activated cytotoxic lymphocytes in vitro. BeWo cells were resistant to the cytolytic effects of the activated lymphocytes despite 24-h exposure and intimate cell-to-cell contact as determined by microscopy. Control target cells, a line of human hepatoma cells, were readily destroyed. Cytotoxicity was measured by determining residual radioactivity of [³H]thymidine-labeled target cells after exposure to activated lymphocytes. Employing the quantitative assay, we confirmed the morphologic results and showed that BeWo and a number of other choriocarcinoma cell lines were resistant to the cytotoxic effects of lymphocytes activated by phytohemagglutinin, pokeweed mitogen, and allogeneic cells in mixed lymphocyte cultures. Moreover, BeWo cells were resistant to injury over a wide range of killer to target cell ratios. Significant killing of the BeWo cells occurred only after prolonged exposure (48 and 72 h) to the activated lymphocytes.

We suggest that one mechanism that may assist the fetus (or a choriocarcinoma) in its immunologic survival is the intrinsic resistance of trophoblast cells to lymphocyte-mediated cytotoxicity.

INTRODUCTION

The survival of the mammalian fetus as a maternal allograft for the duration of pregnancy is a phenomenon that has puzzled transplantation biologists for many years. It is certain, however, that the interface between the maternal circulation and the hybrid fetus is the syncytial trophoblast (1), and it has been shown that trophoblast cells, as opposed to embryonic cells, are capable of surviving allotransplantation (2, 3). Thus, some property of the trophoblast confers upon it the ability to avoid, suppress, or resist immunologic rejection.

Cultured human choriocarcinoma cells exist that share many morphologic, metabolic, and functional characteristics with trophoblast cells of the normal placenta (4). These include the ability to synthesize and secrete human chorionic gonadotropin (hCG)¹ (5), and a surface coating of glycoprotein (6) that has recently been shown to contain hCG (7, 8). The availability of these cells, coupled with the existence of techniques for activating lymphocytes, has provided the opportunity to develop an in vitro model for the immunologic relationship between mother and fetus. Our initial studies involve mixing mitogen-activated lymphocytes with cultured choriocarcinoma cells and showing that the choriocarcinoma cells are resistant to lymphocyte-mediated cytotoxicity.

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¹ Abbreviations used in this paper: BeWo, choriocarcinoma cells; hCG, human chorionic gonadotropin; MLC, mixed lymphocyte cultures; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

METHODS

Preparation of mitogen-activated lymphocytes. Peripheral blood lymphocytes were obtained from healthy young adults and cultures established as described previously (9, 10), except that buffy coat erythrocytes were lysed by a 10-min exposure to 0.74% NH_4CL at 37°C. "Killer" cells were generated by exposing 2×10^5 lymphocytes to phytohemagglutinin (PHA-P, Difco Laboratories), at a concentration of 7.5 $\mu\text{g/ml}$, pokeweed mitogen (PWM; Grand Island Biological Co., Grand Island, N. Y.) at a concentration of 66.4 $\mu\text{g/ml}$, and in mixed lymphocyte cultures (MLC) employing normal peripheral blood lymphocytes (10) and cells of the lymphoblastoid cell line NHDL-2, treated with mitomycin C, as stimulating cells (11). Cells exposed to PHA and PWM were grown in microculture plates or Kahn tubes. MLC were carried out in Kahn tubes with 2×10^5 stimulating cells and an equal number of responding cells. The cultures were incubated at 37°C in 5% CO_2 and humidified air for 3 (PHA, PWM) or 7 (MLC) d. As controls, additional lymphocytes were incubated without mitogens for the same period. At the end of the incubation period the cultures were pooled and centrifuged 10 min at 150 g. The supernatant fluids were removed and the cells then resuspended in fresh medium supplemented with 10% human AB serum.

Target cell culture techniques. Choriocarcinoma (BeWo) cells were obtained in 1974 through the generosity of Dr. Roland Pattillo of the Medical College of Wisconsin in Milwaukee, Wis. These cells have been characterized extensively by Pattillo and his colleagues (4–6, 12, 13). The choriocarcinoma cell lines Reid and JEG (14) were obtained in 1976 from Dr. Janice Chou of the National Cancer Institute, Bethesda, Md. The choriocarcinoma line C2Jar and a cell line derived from a hydatidiform mole were obtained from Dr. John Brewer of the Department of Obstetrics and Gynecology, Northwestern University School of Medicine, Chicago, Ill. Control target cells used in cytotoxicity experiments were a line of human hepatoma cells obtained from a tumor in 1972 and maintained by serial passage in our laboratory ever since (15). Although not extensively characterized, they do not synthesize plasma proteins under our culture conditions and thus may represent fibroblasts. They were chosen as controls for these studies by virtue of their known susceptibility to human lymphotoxin (11, 15), and it was assumed that they would be sensitive to direct lymphocyte-mediated cytotoxicity as well. In this regard they are comparable to HeLa or mouse L cells. HeLa cells and a lymphoblastoid cell line NHDL-2 were used as previously described (11). All the cell lines were grown in RPMI 1640 tissue culture medium (glucose concentration, 2 g/liter) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (50 $\mu\text{g/ml}$). At the outset, 2×10^6 cells were inoculated into a 75-cm² plastic tissue culture flask (3024; Falcon Plastics Div., BioQuest, Oxnard, Calif.) and grown to confluence at 37°C in 5% CO_2 and humidified air.

hCG was detected in the cell-free culture fluid of the BeWo cells by the passive hemagglutination-inhibition test employing rabbit anti-hCG serum and hCG-coated sheep erythrocytes (Pregnosticon Accuspheres, Organon Inc., West Orange, N. J.). This test detects hCG in concentrations as low as 0.75 IU/ml and was consistently positive in cell-free culture medium obtained from the BeWo and other choriocarcinoma cells after 48–96 h of culture.

For morphologic studies, both hepatoma and BeWo cells were grown for varying periods of time in eight-chamber tissue culture slides (4808; Lab-Tek Products, Westmont, Ill.), or Falcon tissue culture dishes (3004; 100 × 100-mm dish with

four 35-mm wells). Three day-old cultures of lymphocytes exposed to PHA, PWM, or medium alone were added to the chambers. Observations were made at regular intervals by examining the cultures with an inverted phase microscope or by direct microscopy after removing the medium, gently washing the monolayer once, fixing in absolute methanol, and staining with Giemsa. Photomicrographs were taken as color slides with Tungsten (3200K) Kodak, high-speed Ektachrome film, EHB 135 (Eastman Kodak Co., Rochester, N. Y.), and subsequently printed in black and white.

Assay for cell-mediated cytotoxicity. A cytotoxicity assay was devised, based on the assay of Jagarlamoody et al. (16). This technique is based on the fact that live and growing cells take up [³H]thymidine in direct proportion to their number. Cytotoxicity is detected by a loss of radioactivity from prelabeled cells because of cytolysis and/or detachment from the surface of the culture vessel. In addition, we exploited the ability of the proteolytic enzyme, Pronase (Calbiochem, San Diego, Calif.), to digest dead cells selectively (17, 18).

Individual cultures used in the quantitative cytotoxicity assays were established by exposing both the hepatoma and BeWo cells to 0.25% trypsin for 10 min at 37°C on a rocking platform (Bellco Glass, Inc., Vineland, N. J.). We then added 20 ml of RPMI 1640 with 10% fetal calf serum and pipetted the cells up and down vigorously 20–30 times. Cells that remained adherent to the flask were dislodged by squirting the surface repeatedly with medium from a Pasteur pipette.

8,000 live hepatoma cells in a single cell suspension were then inoculated, in 0.2 ml medium, into the interior wells of a flat-bottomed microculture plate (IS-FB-96; Linbro Chemical Co., Hamden, Conn.), and allowed to settle for 1 h. Because the BeWo cells grow in clumps and islands (Fig. 1), it proved impossible to obtain suspensions of single cells (see Results). To circumvent this problem, 1 ml of cell suspension was diluted 1:2, 1:4, 1:8, and 1:16, and 0.2-ml portions of each were dispensed into the wells of a microculture plate as described above. After 30 min of settling, the cells were inspected using an inverted phase microscope, and the dilution that most closely approximated the density of the hepatoma cells was chosen for use. The stock suspension of BeWo cells was resuspended further, diluted appropriately, and dispensed in 0.2-ml portions into the wells of a microculture plate.

After incubating the cells for 1 h at 37°C, 0.5 μCi tritiated thymidine (5 Ci/mmol, sp act (Amersham-Searle Corp., Arlington Heights, Ill.), [³H]methyl-³H]thymidine) in 0.05 ml medium was added and the cells returned to the incubator for 16 h. Then the tritiated thymidine was washed off the monolayer with medium containing Hank's buffered salt solution (HBSS), 20 mM Hepes buffer (Calbiochem, San Diego, Calif.) antibiotics, unlabeled thymidine (1 $\mu\text{g/ml}$), and 5% FCS (hereafter called "wash medium"). This procedure was automated by employing a 12-channel manifold attached to a multi-channel dispenser (Cooke Engineering Co., Alexandria, Va.) and a second manifold for suction attached to a wall vacuum. After washing, the cells were left in 0.2 ml of the wash medium and incubated at 37°C until used as targets.

Immediately before adding mitogen-activated lymphocytes, the wash medium was gently sucked out of the wells containing the [³H]thymidine-labeled target cells. Because of the difficulty of counting clumped, transformed lymphocytes accurately, 0.2-ml portions of pooled cells were added to target cells such that each target cell culture received the equivalent of one original lymphocyte culture (2×10^5 lymphocytes).

The mixture of lymphocytes and labeled target cells was incubated at 37°C for 24 h. 2 h before harvest, 0.1 ml of 1 mg/ml solution of Pronase (B grade) in RPMI 1640 was added to the wells containing killer and target cells (final

concentration of Pronase being 0.33 mg/ml). The cultures were then aspirated onto glass fiber filter paper (934 AH; H. Reeve Angel & Co., Inc., Clifton, N. J.) with a Multiple Automated Sample Harvester (MASH-I Microbiological Associates, Bethesda, Md.) and washed with 0.9% saline. Disks containing the radioactivity from a single well were cut from the glass fiber strip with a stainless steel spring punch (Sienco Inc., Morrison, Colo.) and the radioactivity estimated by liquid scintillation spectrometry.

The data are presented as means of four or five replicate samples $\pm$ SEM. The average percentage variation for cytotoxicity assays employing the hepatoma cells as targets was 2.6%, and the average percentage variation for assays employing the BeWo cells was 10%. Tests of significance between sample means were performed by *t* tests between the means of unpaired samples.

RESULTS

Morphologic studies. BeWo cells grew readily in RPMI 1640 and synthesized immunoreactive hCG. Microscopic examination at low power showed cells of various sizes which appeared on the slide chambers to grow singly or heaped up in clumps or islands (Fig. 1). Although the cells readily detached from plastic surfaces during trypsinization, many of the cell clumps

could not be dispersed even by vigorous pipetting or vortex mixing. Exposing the cells to higher concentrations of trypsin alone, or combining 0.25% trypsin with 0.1 M citrate or 0.02 to 2.0% EDTA failed to yield a suspension of single cells. Similarly, employing Pronase and cetrimide as described by Stewart and Ingram for dispersing lymphocytes agglutinated by PHA (17) was ineffective. Under identical conditions, the hepatoma cells, which were much smaller, grew to confluent monolayers and were easily dispersed into single-cell suspensions.

The addition of mitogen-activated lymphocytes to the cell cultures for varying periods of time (4–24 h) damaged the hepatoma cell monolayer extensively. This was manifest by the early appearance of lymphocytes adhering to the target cells, after which the targets became vacuolated and pyknotic. The hepatoma cells then detached from the surface of the culture vessel, clearing it. When mitogen-activated lymphocytes were added to cultures of BeWo cells, there was no change in the appearance of the culture. Although the activated lymphocytes were observed to adhere to the BeWo cells, even appearing to invaginate the

FIGURE 1 BeWo cells growing on a chambered tissue culture slide ($\times 200$, Giemsa). Cells appear singly and in an island.

target cell surface (Fig. 2), they did not damage the BeWo cells even after 24 h of intimate contact.

The failure of mitogen-transformed lymphocytes to damage the growing BeWo cells was seen macroscopically after fixing and staining target cells grown in tissue culture cluster dishes. Fig. 3 shows the results of an experiment wherein PHA- and PWM-activated lymphocytes (wells I and III, respectively), as well as fresh medium and medium from the PHA-stimulated lymphocytes (as a source of lymphotoxin), were added to BeWo cells (wells II and IV, respectively). As may readily be seen, there is essentially no difference in the cell density in any of the wells. Fig. 4 shows the macroscopic appearance of hepatoma cell cultures to which PHA-transformed lymphocytes (well I), PWM-transformed lymphocytes (well III), fresh medium (well II), and unstimulated lymphocytes (well IV) have been added. It is readily apparent that the addition of mitogen-activated lymphocytes has produced marked clearing of the hepatoma cell monolayer.

The difficulty in detecting small or even moderate degrees of cytotoxicity in such a system is obvious. For

that reason a quantitative assay for estimating the extent of cell-mediated cytotoxicity was devised.

Validation of pronase digestion as a means of determining cytotoxicity. At the outset it was discovered that repeated washing (3–4 times) of culture wells containing BeWo cells resulted in unacceptably high losses of live cells (29% in an experiment designed to estimate such loss quantitatively). This was contrasted with a loss of only 10% which occurred when the hepatoma cells were similarly washed. Thus, it seemed desirable to devise an assay for cytotoxicity which did not require repeated washing of the BeWo cell monolayer. Pronase, which has been shown by others to digest dead cells selectively while leaving live cells intact (17, 18), seemed ideally suited for use in such an assay.

The fact that Pronase selectively digests injured and dead cells was confirmed in an experiment where live and dead cells (obtained from overgrown cultures) were mixed, labeled with [^3H]thymidine for 18 h, and incubated with Pronase for varying periods of time. The cells were then either examined microscopically

FIGURE 2 Higher power ($\times 430$) view of the interaction of PWM-activated lymphocytes and the BeWo cells. The transformed cells appear to be enmeshed in a network of BeWo cell cytoplasm and cytoplasmic processes.

FIGURE 3 Macroscopic view of BeWo cells growing in the wells of a tissue culture cluster dish. Well I contains BeWo cells to which 1.2×10^6 PHA transformed lymphocytes have been added for 24 h; well II, BeWo cells to which fresh medium has been added; well III, BeWo cells to which 1.2×10^6 PWM-transformed lymphocytes have been added; well IV, BeWo cells to which the medium from the PHA-transformed lymphocytes (as a source of lymphotoxin) has been added.

for their ability to exclude trypan blue dye or harvested and the cell-associated radioactivity determined. The results of this experiment employing both hepatoma and BeWo cells are presented in Table I. It may be seen that by 2 h of incubation the percentage of viable hepatoma cells approaches a maximum of 98–99%, and the percentage of viable BeWo cells approaches a maximum of 90–91%. That the lysis of dead and injured cells did not occur at the expense of live ones (defined by their ability to incorporate [3 H]thymidine in the 16-h incubation) is indicated by the data showing that no significant decrement in the cell-associated radioactivity occurred during the course of the incubation.

Additional studies with [3 H]thymidine-labeled BeWo cells that had been frozen (-70°C) and thawed four times revealed that Pronase treatment solubilized more than 99% of the cell-associated radioactivity. By contrast, similarly frozen and thawed BeWo cells harvested without Pronase treatment retained variable amounts of [3 H]thymidine label, ranging from 6 to 57% of the starting values.

Resistance of BeWo cells to cell-mediated cytotoxicity. When the quantitative assay for estimating cell-mediated cytotoxicity was used to study the interaction of mitogen-activated lymphocytes with hepatoma and BeWo target cells, the results obtained in the morphological studies were confirmed. Table II presents the results of a typical experiment in which control

medium, control lymphocytes, and lymphocytes exposed for 3 d to PHA and PWM were placed in cultures with hepatoma cells and BeWo cells. It may be seen that the activated lymphocytes exert considerable cytotoxic effect upon the hepatoma cells but none whatsoever upon the BeWo cells. A summary of data obtained in 16 similar experiments is shown in Table III. Under the conditions of our assay, BeWo cells are resistant to injury by activated lymphocytes.

The resistance to cell-mediated cytotoxicity of four other choriocarcinoma cell lines was similar to that shown by BeWo cells. A summary of six additional experiments performed with Reid, JEG, C2Jar, a hydatid cell line, and a later passage BeWo is presented in Table IV. As an additional control target, HeLa cells were used and found to be comparably sensitive to the hepatoma cells. With the passage of time, some variation has occurred in the susceptibility of the BeWo cells to injury inflicted by cytotoxic lymphocytes. However, there continues to be marked differences between the sensitive controls and the relatively resistant choriocarcinoma cell lines.

That lymphocytes activated in MLC behave similarly to PHA- and PWM-activated lymphocytes is shown in Table V. In this experiment the lymphocytes of two normal subjects were mixed with each other and with lymphoblastoid cells (NHDL-2) treated with mitomycin C. The lymphocytes activated in both types of MLC

FIGURE 4 Macroscopic view of cultured hepatoma cells growing in the wells of a tissue culture cluster dish. Well I contains hepatoma cells to which PHA-transformed lymphocytes have been added for 24 h; well II contains hepatoma cells to which fresh medium has been added; well III, hepatoma cells to which 1.2×10^6 PWM-transformed lymphocytes have been added; well IV, hepatoma cells to which unstimulated lymphocytes (controls) have been added.

TABLE I
Effect of Pronase on Mixture of Live and Dead Cells

Time of exposure to pronase	Hepatoma cells		BeWo cells	
	Viable*	Residual [³ H]thymidine†	Viable*	Residual [³ H]thymidine†
	h	%	h	%
0	43	64,508±4,812	37	70,797±4,653
1	98	69,825±1,086	40	73,409±4,952
2	98	71,597±2,860	90	70,131±3,649
3	99	72,063±1,653	91	66,486±5,952

* Cells capable of excluding trypan blue.

† Means of quadruplicate cultures±SEM.

exhibit considerable nonspecific cytotoxicity when added to a monolayer of growing hepatoma cells, but little or none when added to the BeWo cells. The reduction in radioactive label which occurred in the presence of 4×10^5 lymphocytes from each individual subject is unexplained.

Attempts to increase the killing of BeWo cells by activated lymphocytes. The effect of varying the ratio of activated lymphocytes to target cells is shown in Fig. 5. In this experiment, lymphocyte-target cell ratio was varied over a range of 12.5:1 to 200:1. Activated lymphocytes damage the hepatoma cells maximally at a 50:1 ratio, and the data describe a curve that is quite typical of this type of experiment. By contrast, the lack of effect on the BeWo cells is manifest throughout the range of lymphocyte:target ratios examined.

Prolonging the exposure time of activated lymphocytes to the target cells was also investigated as a means of augmenting the ability of the lymphocytes to injure the BeWo cells. In these experiments, the activated lymphocytes were left in contact with the monolayers of hepatoma and BeWo cells for 24 (the usual exposure time), 48, and 72 h before the addition of

TABLE II
Resistance of BeWo Cells to Cell-Mediated Cytotoxicity

Culture conditions	Target cells			
	Hepatoma		BeWo	
	Residual [³ H]thymidine*	% Medium control	Residual [³ H]thymidine*	% Medium control
	cpm		cpm	
Medium control	63,006±537	—	69,827±3,586	—
Lymphocytes Control	59,090±1,643	92	74,437±3,352	107
Lymphocytes + PHA	24,144±292	38	74,560±2,625	107
Lymphocytes + PWM	15,675±492	25	71,216±2,850	102

* Mean±SEM; n = 5.

TABLE III
Summary: Resistance of BeWo Cells to Cell-Mediated Cytotoxicity

Culture conditions	Target cells	
	Hepatoma	BeWo
	% Medium control*	% Medium control*
Lymphocytes – control (16)†	89±3	95±3
Lymphocytes + PHA (7)	49±3	98±13
Lymphocytes + PWM (14)	24±3	79±6

* Mean±SEM.

† Number in parentheses indicates the number of experiments.

Pronase and harvest. Table VI shows that prolonging the exposure of PHA-stimulated lymphocytes to the target cells produced insignificant changes in the cytotoxic activity exerted against both types of target cells. The cytotoxic effect exerted by PWM-activated lymphocytes against the hepatoma cells was maximal after 24 h exposure. However, the PWM-activated lymphocytes did increase their cytotoxicity against the BeWo cells when exposure time was prolonged to 48 and 72 h. Nonetheless, the extent of injury to the target BeWo cells is considerably less than to the hepatoma cells.

DISCUSSION

Our experiments represent an attempt to study the interaction in vitro of cultured human choriocarcinoma

TABLE IV
Summary: Resistance of Cultured Choriocarcinoma Cells to Cell Mediated Cytotoxicity

Target cells	Lymphocytes added to target cells* (cytotoxicity expressed as % of medium control)		
	Control	+ PHA	+ PWM
Controls			
Hepatoma	88±2 (6)	22.5±7.5 (4)	16±6.5 (7)
HeLa	107 (1)	—	28 (1)
Choriocarcinoma cells			
Reid	124±27 (4)	113±22 (3)	93±5 (5)
JEG	97±9 (4)	80±7 (4)	94±11 (4)
C2Jar	115±10 (2)	94±38 (2)	99±24 (2)
Hydatidiform mole	108±1 (2)	97±16 (2)	94±7 (2)
BeWo	93±7 (4)	74±12 (3)	77±12 (5)

* Medium control±SEM (n).

TABLE V
*Resistance of BeWo Cells to Cell-Mediated Cytotoxicity
Generated in Mixed Lymphocyte Cultures*

Culture conditions	Target cells			
	Hepatoma		BeWo	
	Residual [3H]thymidine*	% Medium control	Residual [3H]thymidine*	% Medium control
	cpm		cpm	
Medium control	12,113±716	—	27,239±1,488	—
A† + LCL _{MC} §	2,289±174	19	29,509±2,944	108
B† + LCL _{MC}	1,456±149	12	27,814±3,627	102
A + B	2,545±99	22	24,532±1,876	90
A + A	10,494±297	89	19,291±1,720	71
B + B	10,238±722	87	23,111±3,541	85
LCL _{MC}	10,046 [¶]	85	26,248±1,102	96

* Mean±SEM; n = 5; except for medium control where n = 10.

† A and B are normal subjects.

§ LCL_{MC} are lymphoblasts (NHDL-2) treated with mitomycin C.

¶ Test performed with single culture only.

cells and mitogen-activated, cytotoxic lymphocytes. Our hope was to develop a model for the immunologic relationship between a sensitized mother and her fetus or, alternatively, between a woman and her choriocarcinoma.

The BeWo cells used in these studies were developed as a continuous cell line from a choriocarcinoma of the fetal placenta in 1966 by Pattillo and Gey (4) and Patillo et al. (5). When examined by light and electron microscopy, these cells show strik-

FIGURE 5 Cytotoxic effects of human peripheral blood lymphocytes exposed for 3 d to PHA (circles), PWM (squares), and to medium alone as controls (triangles) mixed with BeWo target cells (solid lines) and hepatoma (control) target cells (dashed lines). 8,000 hepatoma cells and a similar number of BeWo cells (see text for details) were cultured, and transformed lymphocytes were added to achieve the ratios indicated on the abscissa. The residual radioactivity found in the hepatoma cells "medium control" was 26,534±504 cpm, and in the BeWo cells medium control was 59,922±2268 cpm.

TABLE VI
*Effect of Prolonging the Exposure of Stimulated
Lymphocytes to Target Cells*

Mitogen	Target cells	Residual [3H]thymidine* after:		
		1 d	2 d	3 d
PHA	Hepatoma	33	35	25
PHA	BeWo	103	126	98
PWM	Hepatoma	10	12	7
PWM	BeWo	109	67	59

* % of the medium control.

ing morphological similarities to normal trophoblast (6–8). When cultured appropriately, these cells accumulate large amounts of glycogen, and synthesize hCG, human placental lactogen, and various estrogenic and progestational steroid hormones (12). Although something is known of the surface properties of these cells (13), to our knowledge, the immunologic properties of these cells have not yet been studied.

Our morphologic experiments revealed that mitogen-activated lymphocytes readily attach to and damage the control target cells. This phenomenon, which has been described by others (19–22), represents the activation of cytotoxic, thymus-derived (T) lymphocytes by PHA (23, 24) and allogeneic cells (25), and bone marrow-derived and T lymphocytes by PWM (25). These same activated lymphocytes appear to come into intimate contact with the BeWo cells (Fig. 2) but fail to damage these targets in any perceptible way. Thus, the resistance of the BeWo cells to lymphocyte-mediated killing cannot be explained by failure of contact between lymphocyte and target cells. It should also be pointed out that BeWo cells depicted in Fig. 2 had been incubated for 24 h and then washed twice before being photographed. Thus it is likely that the contact between lymphocyte and target had been established to the point where a firm adhesion between lymphocyte and BeWo cells existed. Our figures resemble those published by Vandeputte and Sobis (2) who showed that mouse trophoblasts were unaffected in vitro by exposure to spleen cells from specifically sensitized mice.

Our quantitative cytotoxicity assay was modified from that described in 1971 by Jagarlamoodu et al. (16). The addition of Pronase just before cell harvest serves the double purpose of releasing cells from the culture well and selectively digesting injured and dead target cells, thus solubilizing the previously incorporated label. Stewart and Ingram (17) used Pronase previously to digest dead cells and debris before determining nuclear size of PHA-stimulated lymphocytes. Tiilikainen et al. (18) used Pronase to separate genetically

different lymphocytes in vitro after one cell population had been lysed with cytotoxic antiserum and complement.

In our system, using Pronase avoids the pitfall inherent in many cytotoxicity assays of overestimating cytotoxicity resulting from the possible release of live cells from the surface of the culture vessel during the course of the experiment. Our technique also obviates the necessity for repeatedly washing the monolayer with its attendant nonspecific loss of live cells. The cultures may then be prepared directly for liquid scintillation counting by an automated sample harvester. In this way, the time required to perform the assay is markedly reduced, and the number of samples that may be handled simultaneously increased. In addition, this technique reduces the amount of radioactive wash solutions generated in each experiment and thus reduces the volume of radioactive waste for disposal. It must be pointed out that the quantitative aspects of our findings must be regarded as estimates. Like others, (14), we have experienced difficulty in obtaining suspensions of single BeWo cells after trypsinization of the cultures. However, the quantitative cytotoxicity assays were always monitored by examining the wells by phase microscopy before harvesting, and the appearance of the cultures consistently correlated well with the numerical results obtained from the determination of residual isotope.

The experiments carried out employing quantitative cytotoxicity assays confirmed that BeWo cells were resistant to cell-mediated lysis, whereas the control target cells were lysed readily. In addition, we found that varying the number of activated lymphocytes applied to the hepatoma target cells from 100,000 to 1.6 million produced maximum cytotoxicity at 50:1 lymphocyte:target cell ratios. By contrast, there was no detectable killing of BeWo cells over the whole range of lymphocyte:target cell ratios. Prolonging the time for lymphocyte-target cell interaction failed to augment cytotoxicity of PHA-stimulated lymphocytes. However, when PWM-activated lymphocytes were used as killers, we observed significant injury to the BeWo cells for the first time in any of these studies. It is still clear, however, that the BeWo cells exhibit much greater resistance to lymphocyte-mediated killing than do the hepatoma target cells. In experiments using additional choriocarcinoma cell lines Reid and JEG (14), C2Jar, a hydatidiform mole, and HeLa cells as control targets, we obtained results identical to those obtained earlier with the BeWo cells. Thus, the resistance to lymphocyte-mediated cytotoxicity shown by BeWo cells appears to be characteristic of choriocarcinoma cells generally.

The observations of Allison and Ferluga (26) concerning the mechanism of lymphocyte cytotoxicity perhaps offer an explanation for our results. These investi-

gators noted that proteinase inhibitors suppressed the ability of lymphocytes and isolated lymphocyte plasma membranes to lyse tumor cells. They concluded that a lymphocyte membrane proteinase participated in the lytic reaction. Thus, cells whose surfaces are relatively resistant to the effects of proteolytic enzymes would be expected to be more resistant to immune lysis. The data presented in Table I describing the time required for Pronase treatment to clear dead cells indicates that BeWo cells are digested more slowly and less completely than the hepatoma cells. This may be related to the inability of trypsin treatment of growing BeWo cells to break up clusters and produce suspensions of single cells. In addition, we recently have shown that exposure of target cells to trypsin before adding activated lymphocytes augments the killing of hepatoma cells but not BeWo cells.

The resistance shown by BeWo cells to enzyme treatment and to cell-mediated cytotoxicity may well derive from their coating of acid mucoprotein (6). Normal trophoblast is similarly coated, and hCG, hyaluronic, and neuraminic acids have been identified as constituents of the coat (7, 8, 27). It has been known for a number of years that neuraminic acid can block the action of trypsin and it has been speculated that other sugar moieties may do so as well (28). Although attempts to modify the antigenicity of trophoblast tissue with neuraminidase have produced conflicting results (29-32), it is possible that additional substances, e.g., the hyaluronic acid, or hCG, which recently has been shown to inhibit lymphocyte-mediated cytotoxicity (33), may be involved. Clearly, studies of the biochemistry of the surfaces of BeWo cells might provide considerable insight into their unique immunologic nonreactivity.

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