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Modification of Surface Membrane Antigens by Trypsin[1] (38674)

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The appearance of tumor-specific transplantation antigens (TSTA) on the cell surface is characteristic of virus-transformed fibroblasts (1). These transformed cells are capable of being agglutinated by plant lectins, such as wheat germ agglutinin (2–4) and concanavalin A (5), while normal cells remain unaffected. However, when normal fibroblasts are treated with trypsin they likewise are agglutinated in the presence of these lectins (6, 7). It has been suggested that this increased agglutination of trypsin-treated normal cells may result from changes in the arrangement of the surface lectin receptors (6–8). Although the immunologic implications remain to be clarified, it has been reported that treatment of virus-induced rat lymphoma cells with trypsin resulted in a significant increase in the number of virus-associated surface antigens (9). In addition, studies using specific antisera against plasma membrane antigens have revealed alterations in surface antigenic sites following treatment with trypsin (10, 11).

The induction of delayed-type hypersensitivity, employing either cell-free homogenates (12, 13) or partially purified plasma membranes (14, 15) has also demonstrated the presence of virus-induced antigenic determinants on the surface of transformed cells. A foot pad swelling assay for delayed hypersensitivity was utilized in the present study to examine alterations in antigenicity of normal and polyoma virus-transformed mouse fibroblasts following mild treatment with trypsin. The data presented indicate that enzymatic treatment of cultured 3T3 fibroblasts resulted in antigenic modifications similar to those found in polyoma virus-transformed 3T3 cells.

Materials and Methods. Female ICR mice (CD-1 strain, Charles River Laboratories, Wilmington, MA), 8–10 wk old prior to immunization, were used in this investigation. Previous studies (14, 15) indicated that BALB/c mice did not develop delayed hypersensitive reactions against either intact PY-3T3 cells or PY-3T3 plasma membrane preparations, while NIH Swiss and ICR/CD-1 mice exhibited cellular immune responses over a range of PY-3T3 dosages.

Two lines of fibroblasts were employed: 3T3, an established cell line of BALB/c fibroblasts, and PY-3T3, an established line of polyoma-transformed 3T3 fibroblasts. All cultures were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 10% calf serum and 0.9% antibiotics solution (10,000 units penicillin and 10,000 μg streptomycin/ml, Grand Island Biological Co.). The PY-3T3 and non-transformed 3T3 cell lines showed the typical appearance of mouse fibroblasts and were free from mycoplasma.

Cells were grown at 37° and harvested five days after subculturing. Preparations were obtained for immunization employing either a mechanical or enzymatic procedure. Control cultures of PY-3T3 (*n*-PY-3T3) and 3T3 cells (*n*-3T3) were harvested by scraping them off the glass with a sterile rubber policeman and transferring the suspensions into sterile screw-cap tubes, which were then centrifuged at 800 *g* for 10 min and washed three times with 0.15 *M* phosphate buffered saline (PBS), pH 7.0. The washed cells were stored at −20° until standardized for the immunization regimens. Other cultures (*t*-PY-3T3 and *t*-3T3) were first treated with 0.001% twice crystallized trypsin (Calbiochem) before harvesting. Five ml (10 μg/ml) of trypsin was added to the cultures, gently swirled for approximately 10 sec, and poured off. The cultures were then incubated at 37° for 3

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min, at which time medium containing calf serum was added to dilute the effect of the trypsin. The cells were then collected and washed as described above.

Cell suspensions were diluted in PBS to a concentration of 3×10^5 cells/ml (2%) using standardized turbidimetric measurements with a Klett–Summerson colorimeter. The cell suspensions were diluted 1:2 in complete Freund's adjuvant (Difco Laboratories) and stable emulsions formed. All experimental animals were immunized subcutaneously in the nape of the neck with 0.2 ml of the final preparations. The immunization and challenge regimens for each group of mice are presented in Table I.

At 7 days postimmunization, mice were foot pad tested to detect delayed hypersensitive reactions (14, 16). Preliminary experiments had determined the 7-day rest interval as the optimum time for elicitation of the response to cultured PY-3T3 cells. Cellular immune reactions were demonstrated by measuring the extent of pad swelling following challenge in one hind foot pad with 0.05 ml of an appropriate 1% suspension (2 mg protein/ml) prepared in sterile physiological saline (Table I), as compared to a control hind pad injected with an equal vol of sterile physiological saline. The thickness of the feet was measured to the nearest 0.01 mm immediately before injection and at specific intervals after challenge (4 and 24 hr) by means of a calibrated caliper gauge (Oditest, Model Odi OOT, H. C. Kroplin, Schlustern, Hessen, Germany). The percentage and absolute differences between the experimental and control foot pads were calculated for individual mice and the Student *t* test employed to determine statistical significance of the data. A reaction was considered positive only when swelling in the experimental foot was at least 20% greater than that measured for the saline control.

Results. The foot pad reactions observed 24 hr after challenge of experimental mice with specific cell suspensions are summarized in Table I. All mice immunized with *n*-PY-3T3 cells gave positive delayed hypersensitive responses when challenged with the homologous antigen (Group A). In contrast, none of the animals immunized with *n*-3T3 cells responded to foot pad challenge with a similar cell preparation (Group I). However, when the nontransformed cells were treated with trypsin prior to harvest, they were subsequently capable of stimu-

TABLE I. REGIMENS FOR EXPERIMENTAL GROUPS OF MICE AND DELAYED HYPERSENSITIVE FOOT PAD SWELLING REACTIONS 24 HOURS AFTER CHALLENGE.

Group	Antigen		No. Mice positive/Group	Avg %[c] (Range)
	Immunizing	Challenge		
A	*n* PY-3T3[a]	*n* PY-3T3	20/20	26.9 (23.4–31.0)
B	*t* PY-3T3[b]	*t* PY-3T3	0/27	2.4 (1.0–7.8)
C	*n* PY-3T3	*t* PY-3T3	0/16	2.1 (1.0–5.2)
D	*t* PY-3T3	*n* PY-3T3	0/28	2.7 (1.0–9.8)
E	*n* PY-3T3	*n* 3T3	0/10	10.8 (1.5–13.3)
F	*n* 3T3	*n* PY-3T3	0/9	9.6 (6.6–13.4)
G	*n* PY-3T3	*t* 3T3	0/19	2.9 (0.0–5.9)
H	*t* 3T3	*n* PY-3T3	25/28	25.8 (20.9–29.1)[d]
I	*n* 3T3	*n* 3T3	0/12	1.4 (1.0–2.0)
J	*t* 3T3	*t* 3T3	20/20	24.8 (20.0–26.9)
K	*n* 3T3	*t* 3T3	0/26	2.0 (0.5–3.2)
L	*t* 3T3	*n* 3T3	0/22	4.2 (1.0–12.0)

[a] *n* = Cells harvested by scraping from glass.

[b] *t* = Cells harvested by treatment with trypsin.

[c] Percent difference in swelling between experimental and control foot pads: 100 (experimental-control/experimental).

[d] Denotes average percent and range for the 25 mice demonstrating positive foot pad swelling reactions. The individual values for the mice which were negative were 1.3, 25. and 16.0%, respectively.

lating positive foot pad responses when mice were challenged with either *t*-3T3 (Group J) or *n*-PY-3T3 cells (Group H). Similarly immunized animals failed to respond when challenged with *n*-3T3 fibroblasts (Group L). When other groups of mice were immunized and tested with a number of combinations of normal and trypsin-treated cells, no significant differences were detected between the swelling of experimental and saline-control foot pads.

Similar experiments were performed employing PY-3T3 and 3T3 cells that were treated with a crude trypsin preparation (Grand Island Biological Co.) routinely employed in subculturing these cell lines. Five ml of 0.25% trypsin was added to the culture layers, swirled, and poured off. The cultures were incubated at 37° for 5 min, and medium containing calf serum subsequently added. Groups of mice were immunized and challenged with washed preparations of trypsin-treated or mechanically-harvested cells according to the regimens given in Table I. Following this enzyme treatment, delayed hypersensitive responses were observed in groups corresponding to those described in the table.

Although the data are not depicted in Table I, groups of unimmunized animals challenged with the various cell preparations did not demonstrate significant foot pad swelling reactions. Also, the uniform lack of positive responses at 4 hr after challenge in all groups implied a negligible role for an Arthus-type of humoral reaction in this system.

Discussion. Treatment of cultured PY-3T3 and 3T3 cells with trypsin altered their antigenicity. Whereas normal BALB/c 3T3 fibroblasts were not able to induce a specific delayed hypersensitive reaction, injection of mice with *t*-3T3 cells stimulated positive responses in animals challenged with a homologous suspension. This reaction was shown to be specific for altered surface antigenic determinants when similarly immunized mice did not develop detectable foot pad reactions after challenge with *n*-3T3 cells. Evidently, the surface antigenicity of the normal fibroblasts was substantially modified as a result of exposure to this proteolytic enzyme. Of special significance was the finding that mice initially administered *t*-3T3 cells elicited significant delayed dermal reactions when challenged with *n*-PY-3T3 cells (Group H). The apparent ambiguity in the results observed between Groups G and H has two possible explanations. First, differences in antigen concentration of the two cell preparations (*n*-PY-3T3 and *t*-3T3) could account for the lack of positive responses in Group G. A second possibility involves the antigenic determinants on the cells that elicited the delayed response. Mice given *n*-PY-3T3 cells were believed to be responding to virus-specific transplantation antigens (VSTA) present on the cell surface (14, 15). Numerous studies have indicated that cell-specific fetal antigens are also exposed after virus-induced transformation (17–20). Mild proteolytic enzyme treatment may have exposed similar cell-specific antigenic determinants in the present investigation. Consequently, mice immunized with *n*-PY-3T3 and challenged with *t*-3T3 cells would exhibit negative reactions because of the lack of VSTA on the surfaces of *t*-3T3 cells. In contrast, animals sensitized to the cell-specific antigens on the *t*-3T3 fibroblasts would exhibit delayed responses when challenged with an appropriate dose of similar immunogens present on *n*-PY-3T3 cells. Specific antisera to the cell preparations are being employed in immunofluorescence studies in order to distinguish between the two explanations.

Previous reports have demonstrated that Swiss mice can show delayed-type immune reactions to surface antigens induced by polyoma virus, but do not respond appreciably to normal BALB/c 3T3 histocompatability antigens (14, 15). Thus, it appears that animals immunized with trypsin-treated 3T3 fibroblasts may have responded to newly exposed or altered surface determinant sites, which exhibited antigenic cross-reactivity to immunogens present on untreated polyoma-transformed 3T3 fibroblasts. In reciprocal studies, treatment of cultured PY-3T3 cells with the proteolytic enzyme abrogated the ability of this cell line to elicit a delayed hypersensitive reaction.

Numerous investigations have demonstrated that following mild treatment with proteolytic enzymes, cultured fibroblasts reacted to certain plant lectins in a manner similar to fibroblasts transformed by oncogenic viruses (21–23), indicating biochemical alteration of the plasma membrane. Several other studies have specifically investigated the immunologic alterations of cells treated with trypsin. Treatment of viable normal human lymphocytes with this enzyme was shown to expose previously undetected HL-A surface antigenic sites (10). In addition, trypsinization of normal human diploid cells (WI-38 and MRC 5) resulted in the appearance of complement-fixing activity between the treated cells and an immunoglobulin prepared against a HeLa cell antigen (24). Similarly, when rat lymphomas induced by the Gross leukemia virus were treated with 0.1 mg/ml trypsin for 3 min or longer, a threefold increase in the number of virus-surface antigens was detected; this phenomenon was not observed after treatment with papain or neuraminidase (9). The apparent discrepancy between the above study and the findings in the present report could be due to: (a) different concentrations of trypsin employed in the two investigations; (b) the cell lines were not similar; (c) a DNA virus was utilized in one study and a RNA virus in the other, or combinations of the above.

This study has investigated surface immunologic alterations resulting from treatment of polyoma virus-transformed cells with a proteolytic enzyme and extends the information reported with plant lectins. Studies are now in progress to examine the immunologic activity of the trypsinates and to characterize the cross-reacting surface immunogens of the enzyme-treated 3T3 cells and the virus-transformed PY-3T3 fibroblasts.

Summary. Cultured 3T3 and PY-3T3 mouse fibroblasts were subjected to mild treatment with twice crystallized trypsin and their immunogenicity tested in ICR/CD-1 mice. Surface antigenic modifications were observed using the foot pad swelling assay for detection of delayed hypersensitive responses. Mice immunized with trypsin-treated 3T3 cells showed cell-mediated immune reactions following challenge with either homologous antigen or untreated PY-3T3 fibroblasts. In contrast, treatment of the virus-transformed cells with trypsin decreased their antigenicity as determined by this *in vivo* assay.

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