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Further studies on the mitogenic and immune-modulating effects of plasminogen activator

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Summary. Plasminogen activator (PA), an enzyme which is secreted in large quantities by certain types of tumour cells, is an apparent lymphocyte mitogen. Treatment of mouse spleen cells with anti-Thy-1·2 serum plus complement prevents responsiveness, indicating that T cells are being stimulated to divide. In addition to the foregoing, we have shown that PA has the capacity to interfere with certain types of cell-mediated cytotoxicity and natural killer cell reactions in which tumour cells are employed as targets. These results suggest that PA may play an immune-modulating role which may be of importance in tumour self-defense.

INTRODUCTION

In a previous communication (Cohen, Israel, Spiess-Meier & Wainberg, 1981), we showed that the enzyme plasminogen activator (PA) could function as an apparent lymphocyte mitogen. This proteolytic enzyme, which catalyses the conversion of plasminogen to plasmin, has been shown to be secreted at high levels both by tumour cells and virus-transformed cells (Goldberg, 1974; Unkeless, Gordon & Reich, 1974). Various investigators have postulated that the release of PA by tumour cells and its subsequent effect on cell surface proteins could account for the broad spectrum of antigenicity found in tumour tissue (Reich, 1974). Others have argued that PA probably plays a major role in tumour invasiveness and/or metastasis (Rifkin & Crowe, 1977; Ossowski, Biegel & Reich, 1979).

In our previous report, we speculated that the secretion of PA by tumour cells could serve as a means of tumour self-defense in the face of immune attack. Conceivably, released PA or enzymes with PA-like specificity might cause attacking lymphocytes to divide non-specifically and thus divert them from their task of tumour cell destruction. As in the case of lectins, such as phytohaemagglutinin (PHA), such non-specific proliferation could be followed by death, or at the least, may render immune lymphocytes ineffective in combating tumour growth.

The present research was designed to test the hypothesis that PA might play an immune-modulating role, insofar as the lymphocyte-mediated killing of tumour target cells is concerned. Commercially available, highly purified, PA, in the form of urokinase was used in most of the experiments described here. In addition to testing the effect of PA on specific cell-mediated cytotoxicity reactions, we also investigated

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the influence of this enzyme on target cell destruction mediated by natural killer cells (Kiessling, Klein & Wigzell, 1975; Herberman, Nunn & Lavrin, 1975). These results indicate that PA can significantly impair the ability of lymphocytes from each of two species, chickens and mice, to lyse tumour cells. We further show that, in the mouse the mitogenic effect of PA on lymphocytes is apparently restricted to T cells.

MATERIALS AND METHODS

Lymphocyte stimulation assays

Lymphocyte suspensions were prepared from mouse spleen as previously described (Israel, Yu & Wainberg, 1979), and diluted to a concentration of 10^6 cells/ml in medium RPMI containing 5% foetal calf serum (FCS). Cultures containing 0·25 ml of this suspension were incubated in microwells (Microtiter plates, Flow Laboratories, Rockville, Md.) in the presence or absence of stimulant (50 μl) for 72 hr at 37°. Tritiated thymidine [^{3}H]-TdR; New England Nuclear, Boston, Mass.) (0·2 μCi/well) was added to the wells for the final 16 hr of incubation, after which the samples were processed by trichloroacetic acid precipitation. Cell counts on stimulated samples were routinely made to ensure that measurement of incorporated isotope reflected lymphocyte blast formation and proliferation. Lymphocyte stimulation indices were calculated as the ratio between amounts of radioactivity incorporated in the presence or absence of mitogenic stimulus. All tests were carried out using at least four replicate samples, and standard errors were determined for each mean value obtained. Concanavalin A (Con A, Difco, Detroit, Mich.) was employed as stimulus in some experiments at a concentration of 4 μg/ml. Purified plasminogen activator (PA) was purchased as urokinase, mol. wt. 35,000 (Collaborative Research, Waltham, Mass.; 260,000 CTA units/mg protein) and employed at concentrations ranging between 5 μg/ml and 40 μg/ml.

Cellular depletion experiments

In some cases, specific antisera were employed for the purpose of selectively eliminating various cell populations that might be responsible for mitogenic responsiveness to PA. For the elimination of T cells, mouse spleen cell suspensions (2×10^7 cells/ml) were incubated for 45 min at 37° in a 1:20 dilution in medium of anti-Thy-1·2 serum, kindly supplied by Dr John G. Ray, National Institutes of Health. Bethesda, Md. After being washed by centrifugation, the cells were resuspended in RPMI medium containing a 1:10 dilution of rabbit complement (Cederlane Laboratories, Hornby, Ontario) and reincubated for a further 45 min at 37°. After being washed again, the cells were resuspended in supplemented RPMI medium at a concentration of 10^6 cells/ml, and used as described. A similar protocol was followed for the lysis of B cells, except that a commercial preparation of rabbit anti-total mouse immunoglobulin (Miles Laboratories, Elkhart, Ind.) was employed. Normal mouse and rabbit sera served as negative controls in these experiments and were without effect.

Natural killer cell activity

In the case of mouse cells, these studies were conducted using the YAC-1 mouse lymphoma line (Cikes, Friberg & Klein, 1973) as target. These cells derive from a Moloney leukaemia virus-induced neoplasm, and were kindly supplied by Dr J. Gordon, McGill University, Montreal, Canada. They were maintained in our laboratory as a suspension culture in RPMI medium, supplemented with 10% foetal calf serum. For the purpose of labelling, YAC-1 cells (10^7 cells/ml) were incubated for 30 min at 37° in RPMI medium containing 250 μCi of ^{51}Cr (sodium chromate, New England Nuclear, Boston, Mass.). The cells were then washed three times by centrifugation and resuspended to a concentration of 5×10^5 cells/ml. Natural killer (NK) assays were performed in 11×75 mm plastic test tubes (Falcon Plastics) in total volume of 0·2 ml supplemented RPMI medium. Effector cells were obtained from C3H mouse spleen and were employed at various effector:target ratios. A total of 25,000 ^{51}Cr-labelled target cells were used per tube. All experiments were performed using four replicate samples. Reactions were carried out for 4 hr at 37°, following which 2 ml of medium were added and the samples centrifuged. The amounts of radioactivity in the supernatant and pellet were then measured in a gamma counter.

In the case of chicken cells, the target employed was the LSCC-RP9 line of avian leukosis virus- (RAV-2 strain) producing cells, kindly made available by Dr W. Okazaki, Regional Poultry Research Laboratory, U.S. Department of Agriculture, East Lansing, Mich. (Sharma & Okazaki, 1981). Chicken spleen cell suspensions were prepared as previously described (Israel *et al.*, 1979). Chicken NK assays were performed in 0·2 ml total volume RPMI medium (supplemented with 10% foetal calf serum), as described above for the

mouse NK system. The only difference is that a total of 10,000 chicken target cells were used per assay. Chickens employed in these studies were specific pathogen-free birds purchased from the breeding colonies of Institut Armand Frappier, Laval, Quebec.

For both mouse and chicken NK assays, the percentage of ^{51}Cr released was calculated as follows: % lysis = [(c.p.m. released in presence of spleen cells) − (c.p.m. released spontaneously)/(c.p.m. released maximally) − (c.p.m. released spontaneously)] × 100.

Spontaneous ^{51}Cr release was determined in control tubes without effector cells and varied between 12% and 26% of that amount of label which could be released maximally. Maximal ^{51}Cr release was determined by lysing labelled target cells in detergent (Triton X-101), and measuring the radioactivity of the supernatant after the pelleting of cell debris. The presence of plasminogen activator in culture fluids did not affect levels of spontaneous or maximal ^{51}Cr release.

Specific cell-mediated cytotoxicity

These studies were also performed using the LSCC-RP9 line of avian leukosis virus-producing cells. Since these cells express viral envelope glycoproteins at their surface, they are susceptible to specific lysis by the sensitized lymphocytes of chickens that have been inoculated with either avian leukosis or sarcoma viruses (Wainberg, Yu & Israel, 1979). In our study, sensitized lymphocytes were obtained from the peripheral blood of 9 week-old chickens that had been injected 3 weeks previously with the PrA strain of avian sarcoma virus. The conditions employed in our laboratory for viral growth and inoculation have been described. The use of a limiting virus inoculum has been shown to give rise to a strongly sensitizing, transient tumor, at the site of inoculation, which ultimately regresses (Wainberg *et al.*, 1979). Peripheral blood lymphocytes were harvested from donor chicken blood following centrifugation on Ficoll–Isopaque gradients as previously indicated (Boyum, 1968). These cells were utilized at an effector:target ratio of 100:1. Target cells were labelled with ^{51}Cr as described above, and coincubated with effector cells for 6 hr at 37°. Assays were performed with four replicate samples. The incubation medium consisted of RPMI supplemented with 5% foetal calf serum. These reactions were carried out in microwells (Flow Laboratories; total volume = 0·3 ml), using 10^4 target cells and 10^6 effector cells per well. After 4 hr the cells were transferred to 12 × 75 mm tubes, following which 2 ml of RPMI medium was added and the cells centrifuged. The radioactivity of both pellets and supernatants was counted and the percentage specific ^{51}Cr release calculated as follows: % specific ^{51}Cr release = [(c.p.m. released in presence of sensitized lymphocytes) − (c.p.m. released in presence of normal lymphocytes)/(c.p.m. released maximally) − (c.p.m. released in presence of normal lymphocytes)] × 100.

Pretreatment with urokinase

In some cases, YAC-1 cells or normal mouse splenocytes were preincubated at a concentration of 10^6 cells/ml for 1 hr at 37° in the presence of either 10 μg/ml or 40 μg/ml of urokinase. The cells were then washed three times by centrifugation, resuspended in supplemented RPMI medium, and employed in NK-cell assays, as described above.

RESULTS

Plasminogen activator is a T-cell mitogen

Our initial studies were designed to test whether the mitogenic effect of PA was restricted to T lymphocytes. Accordingly, C3H mouse spleen cells were treated with either anti-Thy 1·2 serum and complement or anti-mouse immunoglobulin plus complement, as described in Materials and Methods, for the selective removal of T and B lymphocytes, respectively. The surviving population was then examined for its ability to be mitogenically stimulated by urokinase. The results (Table 1) show that untreated splenocytes as well as B-cell-depleted spleen cells were able to respond to this stimulus. In contrast, splenocytes that had been depleted of T lymphocytes could not. This indicates that a T-cell population was being stimulated under the circumstances tested.

Effect of plasminogen activator on natural killer (NK) cell activity and on specific cell-mediated cytotoxicity

We next sought to determine whether the presence of PA in culture fluids could interfere with NK-mediated and with specific lymphocyte-mediated cytotoxicity reactions. These studies were carried out using spleen cells of each of two species, mice and chickens. The results of Fig. 1 show that the ability of normal mouse C3H splenocytes to lyse YAC-1 target cells was inhibited in the presence of each of two concentrations

Table 1. Plasminogen activator-driven mitogenesis of mouse lymphocytes

Exp.	Concentration of urokinase used (μg/ml)	Stimulation index	P*	Stimulation index after treatment with anti-Thy-1.2 serum	P	Stimulation index after treatment with anti-immunoglobulin	P
1	5	1·17±·12†	NS‡	1·09±·17	NS	1·21	NS
	10	0·95±·08	NS	1·13±0·9	NS	1·30	NS
	20	2·43±·31	<0·05	1·06±·14	NS	2·19	<0·05
	40	3·78±·40	<0·01	1·36±·20	NS	2·86	<0·05
2	5	0·92±·11	NS	0·98±·11	NS	1·16	NS
	10	1·51±·19	<0·05	1·16±0·7	NS	0·87	NS
	20	2·37±·18	<0·01	1·29±·13	NS	1·99	NS
	40	4·20±·52	<0·01	1·12±·14	NS	3·82	<0·01

* Probability of significant difference from unstimulated culture; Student's *t* test.
† ±Standard error of the mean.
‡ Not significant.

of urokinase, 40 μg/ml and 20 μg/ml. This was true at each of two different effector:target ratios, i.e. 100:1 and 50:1.

Similar results were obtained in the case of an avian NK assay, in which spleen cells from normal chickens were used to lyse targets of the LSCC-RP9 line of avian leukosis virus-producing cells. In this instance, PA was able to inhibit NK activity at concentrations of both 40 μg/ml and 20 μg/ml, when effector:target ratios of either 40:1 or 20:1 were employed. At a lower effector:target ratio, no cell killing was observed either in the presence or absence of urokinase (Fig. 2).

Finally, the LSCC-RP9 line was also used as a target

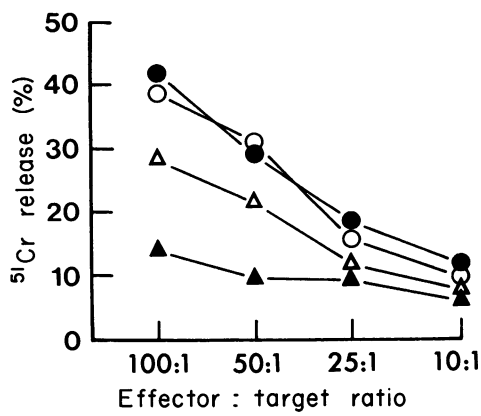

Figure 1. Effect of urokinase on murine NK activity. Concentrations of urokinase employed were (●), 0 μg/ml; (○), 5 μg/ml; (△) 20 μg/ml; (▲) 40 μg/ml. Differences between levels of ^{51}Cr release between control and experimental samples were significant at $P<0·05$ for treatment with urokinase at either 20 μg/ml or 40 μg/ml; paired *t* test.

in a specific cell-mediated cytotoxicity assay, in which the peripheral blood lymphocytes of avian sarcoma virus-injected chickens served as effectors. The results of Table 2 reveal that the inclusion of urokinase (40 μg/ml or 20 μg/ml) in the culture medium was inhibitory to this reaction as well.

Effect of pretreatment with PA of either effector or target cells on NK-mediated cytotoxicity

In order to assess whether PA was interfering with target cell killing as a result of interaction with either target or effector cells, the following experiment was carried out. YAC-1 cells or normal mouse splenocytes were preincubated for 1 hr at 37° in the presence of either 10 μg/ml or 40 μg/ml of urokinase. After being washed and resuspended, the cells were employed in NK assays as described. The results (Table 3) reveal that pretreatment of the YAC-1 target did not affect their susceptibility to lysis by untreated effector cells. On the other hand, when normal mouse spleen cells were pretreated with 40 μg/ml, but not 10 μg/ml of urokinase, their ability subsequently to lyse untreated YAC-1 cells was diminished by as much as 55%. This result suggests that the inhibitory activity of PA in the NK assay is directed against effector rather than target cells.

DISCUSSION

In a previous study (Cohen *et al.*, 1981), we showed that PA can function as an apparent lymphocyte mitogen. At that time, we demonstrated that mitogenic activity

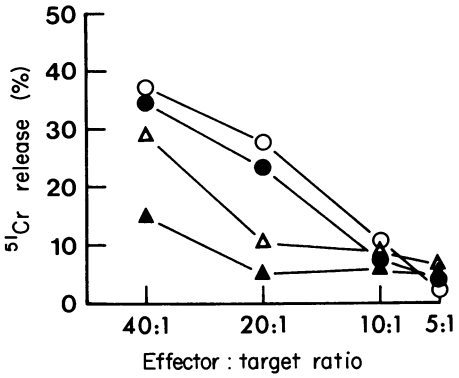

Figure 2. Effect of urokinase on avian NK activity. Concentrations of urokinase employed were: (●) 0 μg/ml; (○) 5 μg/ml; (△) 20 μg/ml; (▲) 40 μg/ml. Differences between levels of ^{51}Cr release between control and experimental samples were significant at $P<0{\cdot}01$ for 40 μg/ml urokinase and $P<0{\cdot}05$ for 20 μg/ml urokinase; paired t test.

Table 2. Effect of plasminogen activator on specific cell-mediated cytotoxicity

Target cells*	Concentration of urokinase (μg/ml)	Specific ^{51}Cr release (%)	P†
N-CEF	—	3·1	
LSCC-RP9	—	23·5	<0·01
N-CEF	5	2·4	NS‡
LSCC-RP9	5	20·8	<0·01
N-CEF	20	—	NS
LSCC-RP9	20	14·7	<0·05
N-CEF	40	4·1	NS
LSCC-RP9	40	3·0	NS

* Target cells were either the LSCC-RP9 line of avian leukosis virus-producing cells or resuspended normal chicken embryo fibroblasts (N-CEF) which served as a negative control.

† Probability of significant difference from control in which sensitized lymphocytes were exposed to N-CEF cells in the absence of urokinase; Student's t test.

‡ Not significant.

was not due to the generation of the serine esterase plasmin from plasminogen, and that mitogenesis could be inhibited by any of several inhibitors specific for PA enzymatic activity. The results of the present communication indicate that this mitogenic activity is restricted to T cells. This result is hardly surprising; it is known that a variety of serine esterases can cause T lymphocytes to divide (Mazzei, Novi & Bazzi, 1966; Kaplan & Bona, 1974).

Table 3. Effect of pretreatment of effector or target cells with plasminogen activator on NK activity

Exp.	Concentration of urokinase used	Cells pretreated*	^{51}Cr release (%)	P†
1	—	—	39·1	
	10	Effector	37·3	NS
	10	Target	35·8	NS
	40	Effector	19·6	<0·05
	40	Target	42·0	NS
2	—	—	34·5	
	10	Effector	37·2	NS
	10	Target	39·6	NS
	40	Effector	15·7	<0·01
	40	Target	32·4	NS

* Either normal mouse C3H splenocytes or YAC-1 cells (i.e. effector and target cells, respectively) were pretreated with urokinase for 1 hr at 37°, as described in Materials and Methods.

† Probability of significant difference from control in which urokinase pretreatment was not performed; Student's t test.

‡ Not significant.

Since PA is secreted in large amounts by certain types of tumour cells and virus-transformed cells (Goldfarb & Quigley, 1978), we reasoned that it might have an immune modulating function. This could provide a means of tumour self-defense in the face of what might otherwise be a successful anti-tumour immune reaction mounted by the host.

In order to test this concept, we included purified PA, in the form of urokinase, in the culture media of several different kinds of cytotoxic reactions. The results show that the presence of PA was inhibitory to the NK-mediated lysis of target tissue, in both a murine and avian model. Others have previously shown that the LSCC-RP9 line of avian leukosis virus-producing cells is susceptible to NK lysis by chicken splenocytes (Sharma & Okazaki, 1981).

PA has been reported by some to play an important role in promoting tumour cell invasiveness and metastasis (Rifkin & Crowe, 1977; Ossowski *et al.*, 1979). More recently, others have argued that entities which are distinct from PA, such as collagenase and laminin, may be involved in metastasis, and that the role of PA in this regard is uncertain (Liotta, Tryggvason, Garbisa, Hart, Foltz & Shafie, 1980).

Our results support the concept that PA may exert an immune-modulating influence inhibitory to NK and specific cell-mediated cytotoxicity reactions. Such

a finding is consistent with but not necessarily related to our independent observation that PA is a T-cell mitogen. Thus, we do not know at present, whether the mechanism through which PA is inhibitory to target cell killing is the same as or related to that responsible for T-cell mitogenesis. Our data on pretreatment of YAC-1 cells or mouse splenocytes with urokinase suggest that the effect of PA is directed against effector and not target cells. Further studies to investigate interactions between PA and immune lymphocytes in the tumour microenvironment are in progress.

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