Ability of the Immunomodulating Dipeptide Bestatin to Activate Cytotoxic Mononuclear Phagocytes

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ABSTRACT

The aminopeptidase inhibitor Bestatin [2S,3R-(3-amino-2-hydroxy-4-phenylbutanoyl)-l-leucine] was tested for both in vitro and in vivo macrophage activation and antitumor activity in various experimental tumor systems including the metastasizing ESb lymphoma system. Cultures of resting macrophages were rendered nonspecifically tumoricidal for the two lymphoma sublines ESb and 721 (ESb-C1 18.1), for the mastocytoma P815X, for LS lymphoma cells, and for proliferating lymphoblasts from various experimental tumor systems including the metastasizing and in vivo macrophage activation and antitumor activity in droxy-4-phenylbutanoyl)-L-leucine] was tested for both in vitro nu) mice treated i.p. with the dipeptide were also stimulated and dependent on the Bestatin dose and appeared about 24 hr after injection of the dipeptide. Bestatin activates macrophages inasmuch as macrophages from homozygous athymic nude (nu/nu) mice treated with Bestatin and rule out the possibility that natural killer cells play a major role in the experiments described.

INTRODUCTION

Over the last decade, considerable emphasis has been placed on modulating the host’s antitumor immune response with substances capable of potentiating tumor-destructive responses or restoring immune competence to the anergic tumor-bearing host. The underlying assumption has been that immunotherapy has the potential to benefit humans in the treatment of cancer. Indeed, at least animals treated with various immunomodulators have been shown to exhibit increased reticuloendothelial activity as well as increased resistance to tumor growth (13, 16).

Most of the agents which enhance host resistance against cancer stimulate mononuclear phagocytes (17). Increasing evidence has been accumulated during the last years that activated macrophages play a crucial role as effector cells in the body’s defense against neoplasia (19, 30). Consequently, interest has been stimulated in the mechanisms of macrophage activation in vitro and in vivo and in agents that can enhance macrophage-mediated destruction of tumor cells. The results thus far show that there is an accumulation of macrophages in regressing tumors (10) and that macrophages are directly cytotoxic to tumor cells when coincubated in cell culture (32). Resistance of animals to tumors can be increased nonspecifically by prior inoculation of several materials, including Mycobacterium tuberculosis strain Bacillus Calmette-Guérin (36), Toxoplasma gondii (20), Corynebacterium parvum (5), double-stranded polyribonucleotides (27), some polyanions (35, 43), synthetic dehydrodipeptides (40), polysaccharides (14), and products from antigen- and mitogen-stimulated lymphocytes (11,37). Macrophages from animals exposed to a wide variety of these immunomodulators but not from unstimulated animals were shown to be cytotoxic for tumor cells in culture, sometimes killing the tumor cells and in other cases inhibiting their multiplication (26). Corresponding to those ex vivo experiments are the in vitro data. Thus, naive resting macrophages in exudates from normal mice, when cultured in the presence of a number of polyanions (2, 41), LPS2 (2), lymphokines (12, 42), and well-defined substances such as lipid A (34) or peptidoglycan of bacterial cell walls (31) can be rendered nonspecifically tumoricidal for various target cells.

The mechanisms by which these agents stimulate macrophages to become cytotoxic are unknown. There are publications on the hypothetical mechanisms of these immunopotentiators (1, 25). However, any better understanding of the pharmacological mechanisms by which the biological and chemical immunomodulating agents affect the cellular components of the immune system and induce tumoricidal or tumoristatic effects will help us in designing a successful combination tumor therapy consisting of a conventional tumor-cytoreductive therapy and immunotherapy.

In this paper, we report on the immunological antitumor response achieved with the chemically defined immunostimulant Bestatin, a potent protease inhibitor of Actinomycetes origin, which is of dipeptide nature (49). Several papers reported that Bestatin augmented the immune resistance to cancer and suppressed the growth of tumors (23, 38, 48). We present further in vivo and in vitro evidence which implicates activated macrophages as one of the most relevant candidates of this Bestatin-induced antitumor resistance.

MATERIALS AND METHODS

Tissue Culture Materials. Tissue culture grade Petri dishes and microtiter plates were obtained from Nunc GmbH, Wiesbaden, West Germany; TCM 199, Dulbecco’s minimal essential medium, RPMI 1640, and fetal bovine serum were from Flow Laboratories GmbH, Bonn, West Germany.

Biochemical Reagents. All salts and reagents were reagent grade dissolved in 1 liter 0.01 M sodium phosphate buffer, pH 7.3.)

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indicated otherwise. Penicillin and streptomycin were from Flow Laboratories; heparin (preservative free) and L-glutamin were obtained from Seromed GmbH, Munich, West Germany; Triton X-100 was from Sigma-Chemie GmbH München, Taufkirchen, West Germany. Bestatin was purchased from Nippon Kayaku Co., Ltd., Chiyoda-ku, Tokyo, Japan, and did not contain endotoxins as detected by the Limulus lysate assay.

Experimental Animals. Female mice of the outbred strain NMR were purchased from Wiga, Sulzfeld, West Germany; female DBA/2, C57BL/6J, and BALB/c mice were obtained from G. L. Bomholtaard, Ltd., Ry, Denmark. The female athymic nude mice (BALB-nu/nu) were purchased from Zentralinstitut für Versuchstiere, Hannover, West Germany.

Target Cells. The following cell lines were used: P815x cells (H-2b), a derivative of the C3H fibroblast cell line L929; the NK-sensitive lymphoma cell YAC-1 and the NK-sensitive human erythroleukemic cell line K562; moreover, mouse lymphocytes from the spleen of DBA/2-H-2b, C57BL/6-H-2b, and CBA-H-2b mice were cultured in RPMI 1640 with 10% fetal bovine serum at a concentration of 4 x 106/ml and stimulated with concanavalin A (2 µg/ml) for 2 days; from the chemically induced DBA/2 lymphoma LS178Y, we used the 2 sublines Esb (H-2b) from Professor Schirmacher (39) and the recloned murine tumor line 721 (H-2b), identical with Esb-CI 18.1 (6). All these cell lines were maintained by serial passage in RPMI 1640 with 10% fetal calf serum, except the K562 cell line which was passaged in Dulbecco’s minimal essential medium.

Macrophage Collection and Culture. Mouse macrophages were obtained peritoneal lavage of the mice with 5 ml of TC 199 containing penicillin and streptomycin (100 units/ml) and heparin (10 IU/ml). Samples (4 ml) of the peritoneal exudate cell suspension containing 0.5 to 1.0 x 106 cells/ml were distributed into 35-mm Petri dishes and incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C for 12 hr to allow attachment of adherent cells. Nonadherent cells were removed by 4 washes with PBS. After the washing, the cells were cultured in TC 199 without serum. Cultures prepared in this way give a sheet of well-spread cells within 24 hr. Resultant monolayers routinely were composed of 95 to 98% mononuclear phagocytes and 2 to 5% small mononuclear lymphocytes (lymphocytes) as determined by the classical criteria (morphology, phagocytosis, nonspecific esterase staining). In all experiments, quadruplicate cultures were used, and biochemical and biological results are expressed as the mean ± S.D.

Macrophage Stimulation. Macrophages were recovered either from normal untreated mice or from mice after parenteral administration of 0.5 ml PBS with or without the dipeptide Bestatin and cultured in 2 ml fresh serum-free TC 199. In the case of in vitro activation, the stimulus was added in various doses; in other experiments, only single doses of stimulus were used, and macrophages were tested after various times of incubation for biological activity.

Cytotoxicity Assay. The lysis of tumor cells was assessed by the release of radioactive chromium from the labeled cells by the method of Brunner et al. (7) and occasionally compared with the trypan blue exclusion. Macrophage cultures were always incubated for 24 hr. Culture medium was then removed; to each Petri dish of cultured macrophages, 2, 4, and 8 x 104 tumor cells in 2 ml TC 199 without serum were added at various effector:target cell ratios (100:1, 50:1, and 25:1 respectively). At these population densities, normal macrophages are not cytotoxic to tumor cells, whereas activated macrophages are. The macrophage:target cell mixtures were then incubated for 6 hr at 37°C. After that incubation, the medium was recovered and centrifuged in plastic tubes, and 1 ml of the supernatant was removed for measurement of the 51Cr activity. The percentage of specific 51Cr release was expressed as

\[
\text{(Label released with macrophages)} - \text{(label released in its absence)} \times 100
\]

\[
\text{(Label released with 1% Triton X-100)} - \text{(label released in its absence)}
\]

Spontaneous chromium release in the absence of added agents ranged between 2.5 and 9% depending on the target cell type.

Statistical Tests. Means ± S.D. were calculated after samples were shown to be homogeneous by calculation of coefficients of variance. The significance of differences was established by Student’s t test.

RESULTS

In Vitro Activation of Tumoricidal Properties in Mouse Peritoneal Macrophages. Mouse peritoneal macrophages were incubated for 24 hr with different amounts of Bestatin ranging from 50 to 200 µg/ml to achieve activation for tumor cytotoxicity. Following incubation with Bestatin, the macrophage monolayers were rinsed thoroughly with TC 199, and 51Cr-labeled tumor cells at various effector:target cell ratios were added. The data shown in Chart 1 demonstrate that a minimum effector cell:target ratio of 50:1 (p < 0.002) was necessary to kill tumor cells. Mouse peritoneal macrophages treated with Bestatin (100 µg/ml) were found to lyse in vitro tumor cells to about 25% when the ratio was increased to 100:1 (p < 0.001). These experiments established also that untreated mononuclear phagocytes and Bestatin alone were not directly cytotoxic to the tumor cells used. Macrophage cytotoxic capacity was accompanied by increased spreading of macrophages on plastic. A similar degree of activation was observed in macrophages from athymic nu/nu mice (Chart 2). Bestatin transformed normal resting macrophages from nude mice into cytotoxic effector cells in vitro to kill ESb tumor cells with optimal activity at 200 µg/ml, although significant (p < 0.001) activity remained at concentrations as low as 50 µg/ml. In contrast, normal resting macrophages had no effect on ESb target cells. We also tested the effect of Bestatin on macrophages from mice of various strains. Mononuclear phagocytes from all strains of mice were used effectively stimulated by the dipeptide. Untreated control macrophages did not induce any cytotoxic effect against ESb tumor cells at an effector:target cell ratio of 100:1. To determine a tumor specificity of this response, the cytotoxic effect of Bestatin-activated macrophages was also tested against various tumor cell lines and phytohemagglutinin-stimulated lymphocytes in culture. Chart 3

Chart 1. In vitro lysis of various tumor cell lines of mouse origin by mouse peritoneal macrophages activated in vitro with Bestatin (100 µg/ml) after 6 hr (---). Untreated cultured macrophages were not cytotoxic against the target cells used (----). Results represent mean values derived from measurements on groups of 4 cultures. Bestatin-treated macrophages showed a statistically significant increase in cytotoxic activity even with an effector:target cell ratio of 25:1 (p < 0.002).

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shows that all the 6 cell types of mouse origin tested were killed effectively, independent of whether they were syngeneic or allogeneic with respect to the macrophage cultures used. ESb tumor cells appeared to be the most sensitive cell type. Neither untreated macrophages nor Bestatin alone in the absence of macrophages affected the tumor cells after an incubation time of 24 hr.

**Induction of Tumor Cytotoxicity in Macrophages from Mice Treated with Bestatin.** Peritoneal macrophages from mice treated in vivo i.p. with Bestatin were large vacuolated cells with a marked propensity to stretch out on glass and plastic compared to normal macrophages. Two hr after cultivation in plastic plates, over 98% of the adherent cells were stimulated macrophages and well spread out, whereas most of the normal control macrophages remained rounded. After longer periods of cultivation, the normal macrophages also started to spread. Morphologically activated macrophages could be recovered from mice 1 day after administration of Bestatin and persisted for at least 10 days.

As shown in Chart 4, unstimulated macrophages did not kill tumor cells as measured by $^{51}$Cr release of labeled cells into the medium, whereas macrophages from Bestatin-treated mice lysed nearly 50% of the tumor target cells used. Macrophages from NMRI mice given i.p. 200-mg/mouse injections of the dipeptide 3 days previously were found to lyse in vitro various tumor cells at an effector cell:target ratio of 100:1. When the ratio of macrophages to tumor cells was reduced to 25:1, still about 10% (p < 0.001) of the target cells were killed. Cytotoxic activity could be found consistently, and the appearance was dose and time dependent.

**Dose Response of Macrophage Activation In Vivo.** Further experiments were performed to test the dose dependency of Bestatin-induced macrophage activation. Since 200 µg of the dipeptide per mouse were able to stimulate cytolytic activity (p < 0.001) in the cultivated macrophages, Bestatin was administered i.p. at doses ranging from 50 to 400 µg/mouse. Macrophages were harvested on Day 3 and tested for cytotoxic activity at an effector:target cell ratio of 100:1. The results summarized in Chart 5 demonstrate that a minimum concentration of 50 µg of Bestatin per mouse was necessary to render mouse peritoneal
of macrophage activation might be absent in athymic nude (nu/nu) mice. However, Bestatin when administered at various doses (50 to 400 µg/mouse) activates macrophages from these animals morphologically and functionally (Chart 7). Macrophages from nude mice were somewhat less cytotoxic for ESb tumor cells at an effector:target cell ratio of 100:1 at 3 days after inoculation of the dipeptide than were macrophages from other mouse strains, but they still were stimulated and could kill more than 40% of the tumor target cells used with a dose of 400 µg/mouse previously injected (p < 0.001). In contrast, the i.p. injection of PBS into nu/nu mice did not produce any significant antitumor activity at that time.

Attempt to Establish the Essential Role of Macrophages for Tumor Cell Destruction. Activated macrophages from Bestatin-treated mice had no preference to be cytotoxic for special tumor cell lines; also normal quickly proliferating blasts were affected to the same degree. Even the ESb lymphoma cell lines used as targets and being totally resistant to NK cells were lysed by Bestatin-activated macrophages. Because Bestatin is not able

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**Activation of Cytotoxic Macrophages by Bestatin**

- **Chart 6.** Kinetics of the appearance and persistence of cytotoxic activity of stimulated macrophages from mice given i.p. injections of 100 µg of Bestatin (A). Statistically significant tumoricidal macrophages were evident after 1 day (p < 0.002). Macrophages were harvested at various time intervals as indicated and tested for cytotoxicity 24 hr later. Macrophages from control PBS-treated mice (•) did not produce any significant lytic activity at any time against ESb tumor cells (effector:target cells, 100:1).

- **Chart 7.** Dose-dependent effect of Bestatin on the in vivo-induced cytotoxicity of macrophages from nude (nu/nu) mice to ESb target cells (effector:target ratio, 100:1). Experimental conditions were identical to those in Chart 4. After treatment with Bestatin (50 µg/mouse), there is a statistically significant increase in cytotoxic macrophage activity (p < 0.01).

- **Chart 8.** Influence of various doses of Bestatin on the in vivo activation of macrophages from NMRI mice to kill spontaneously the NK-susceptible target YAC-1 at various effector:target cell ratios. Experimental conditions were identical to those in Chart 4. (Statistically significant from controls: 50 µg/mouse at effector:target cell ratios of 25:1, 50:1, and 100:1, p < 0.5, p < 0.01, and p < 0.002, respectively; 100 µg/mouse at these effector:target cell ratios, p < 0.01, p < 0.001, and p < 0.0001, respectively).

macrophages tumoricidal (p < 0.001). High levels of the dipeptide (100 to 400 µg/mouse) brought about significantly increased macrophage-mediated cytotoxicity against the various tumor targets used in this experiment. Macrophages from untreated mice did not increase the release of 51Cr into the medium above background level. The cytotoxic effect of macrophages, activated in vivo by Bestatin, was independent of the application route and could be achieved by i.p. or i.v. injections of Bestatin or when given p.o. Also using the genetic model with the C3H/HeJ-C3H/HeN mouse strain pair did not show any significant difference in the macrophage-mediated cytotoxicity induced in vivo by Bestatin. Macrophages from both mouse strains, the LPS-responsive C3H/HeN mice and the LPS-resistant or poorly responsive C3H/HeJ mice were both equally well dose-dependent activated to kill tumor cells, indicating that this activating process by Bestatin is independent of any LPS contamination. (These data are not shown here.)

**Kinetics of the Macrophage-mediated Cytotoxicity Induced by Bestatin.** The time course for Bestatin activation presented in Chart 6 suggests that macrophage activation was optimal after 4 days of dipeptide exposure. Peritoneal macrophages were harvested at various times after i.p. administration of 100 µg Bestatin per mouse from normal NMRI mice and were tested in vitro for their ability to produce cytotoxic activity to kill ESb tumor cells. The lytic activity of the stimulated macrophages was measured on Days 1, 2, 3, 4, 7, 11, and 14 after the i.p. injection. The appearance of cytotoxic macrophages after Bestatin treatment in mice was rapid; tumoricidal macrophages were evident by 24 hr (p < 0.002); macrophages harvested 6 hr after i.p. injection of the dipeptide were not cytotoxic. Optimal activity (more than 50% lysis) was observed on Day 4 and decreased thereafter with further time after Bestatin injection. In contrast, macrophages from control PBS-treated mice did not produce any significant cytotoxic activity at any time.

**In Vivo Activation of Macrophages from Athymic Nude Mice by Bestatin.** If Bestatin activates macrophages via a T-cell-dependent pathway in vivo, it could be predicted that this process

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to induce interferon to stimulate NK cells in the peritoneal exudate, the cytotoxic effects measured were due to cytotoxic macrophages and not to eventually contaminating NK cells. Additionally, macrophages activated in vivo by Bestatin could kill targets susceptible for NK activity such as the YAC-1 tumor cells in the used effectortarget cell ratios as indicated in Chart 8, whereas macrophages from control mice, not treated with Bestatin, could not kill spontaneously the NK cell-susceptible target YAC-1, which seems to be a further argument for the minor role of NK cell-mediated toxicity under the experimental conditions used. A significant increase in the amount of $^{51}$Cr label released from YAC-1 target cells was observed only after 3 days of in vivo dose-dependent stimulation ($p < 0.002$ to $p < 0.001$) with the dipeptide, when compared to the control macrophage cultures.

DISCUSSION

The disappointing results obtained to date in the majority of clinical immunotherapy trials using agents designed to stimulate specific immune responses against tumors have prompted renewed interest in nonspecific mechanisms such as NK cells (15) and activated macrophages as effector cells in immunopotentiator-mediated resistance to tumor growth. Special emphasis has been given to macrophages. In vitro-activated macrophages selectively destroy neoplastic as opposed to normal cells (21, 22). In vivo studies have further implicated activated macrophages as effectors of tumor resistance induced by synthetic and biological immunopotentiators (18, 46). The selective spectrum of cytotoxicity of such macrophages closely agreed with that observed for peritoneal exudate macrophages activated with endotoxin or lymphokines (37, 45, 47).

The use of whole bacteria or their extracts as immunopotentiators in clinical tumor immunotherapy has been hampered by their toxicity. Those undesirable side effects could indeed be reduced by the use of highly purified or even synthesized active bacterial compounds. Those substances have been reported to potentiate humoral antibody responses to various antigens (8, 9) to a similar degree as do the original bacteria, but many of the compounds after being purified now failed to enhance host-mediated cellular immunity (27, 29). This lack of effectiveness may result from the rapid clearance and excretion after parenteral or p.o. administration (33). Thus, much effort has been made to solve this problem by developing special drug device systems. However, there still remained the risk of toxicological side effects such as fever (27), granulomas (35), and allergic reactions (31). In addition, the inability of the body to metabolize some special immunomodulating compounds may impair their general use (28).

In contrast to whole bacteria or their purified components, the dipeptide Bestatin occupies strong immunomodulating activities for humoral and even for the cellular immune response (38, 48) in spite of the fact that it is very quickly metabolized. Moreover, Bestatin has a neglectable toxicity and is not antigenic, is effective when given p.o. or parenterally, and even displays antitumor activity (23, 48). Bestatin has been isolated as a competitive aminopeptidase inhibitor by Umezawa et al. (49). The enzymes aminopeptidase B and leucine aminopeptidase appear on the surface of various mammalian cells. Bestatin can bind to these enzymes and thus inhibit the cell surface activities of the aminopeptidases on macrophages, polymorphonuclear leukocytes, and lymphocytes which might cause some modification in cell function; especially in those involved in immunity (3).

In the case of mononuclear phagocyte stimulation, we could show the capacity of Bestatin to induce macrophage-mediated cytotoxicity. The results demonstrate that mice macrophages obtained by lavage of the peritoneum from normal mice can be rendered tumoricidal to syngeneic, allogeneic, and xenogeneic tumorigenic target cells by incubation in vitro with Bestatin. This drug also activates macrophages in vivo as shown for instance by the ability to kill tumor cells in vitro. Macrophage activation in vitro as well as in vivo by the dipeptide was sharply dose and time dependent. The different doses used for macrophage stimulation in vivo were reflected as a dose-dependent tumoricidal effect in vitro. Activated macrophages appeared after 24 hr, peaked on Day 4, and decreased thereafter.

The cytotoxic effect of activated macrophages cannot be accounted for by the classical immunological reactions. Those are characterized by an inductive phase initiated by antigenic stimulus. This is followed by synthesis of specific antibody or production of specifically sensitized cells directed against the initiating antigens in tumor immunity against tumor-associated antigens. However, target cell destruction mediated by macrophages is apparently governed by a different and non-antigen-specific mechanism. Thus, macrophage activation as early as 24 hr after a single injection of Bestatin should be independent of any specific immune response initiated by the dipeptide. This is supported by the fact that systemic activation of macrophages to the tumoroidal state by Bestatin appears to be independent of the thymus. Macrophage cultures from athymic nude mice were efficient in killing tumor cells, as were macrophages from normal thymic mice. By these findings, a T-cell-mediated mechanism leading to the observed effects seems unlikely. A NK cell-mediated effect cannot be excluded totally at the moment. However, the data showing cytotoxicity of purified macrophage cultures against the NK-susceptible target YAC-1 and against the NK cell-resistant ESb-lymphoma cells implicate under the experimental conditions described activated macrophages and not NK cells as effectors of tumor resistance induced by Bestatin.

The augmented antitumor response produced by the dipeptide Bestatin and mediated by activated macrophages may be important in increase of tumor resistance and in the development of cancer therapy modalities. We believe that Bestatin offers particular promise for future therapeutic efforts to modify macrophage functions. The bulk of evidence to date indicates that, with immune regulating agents, particularly with macrophage activators, a beneficial therapeutic response is achieved at least in experimental animal models. Based on those animal studies, recent clinical trials using Bestatin as an immunoregulating agent (4, 24, 44) indicate that a beneficial effect in tumor patients also might be achieved.

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