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Restoration of impaired immune functions of aged animals by chronic bestatin treatment

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Summary. An attempt to correct the state of immunodeficiency in old age was made by repeatedly injecting a chemically defined immunostimulating agent, bestatin, to 16 month old (C57Bl/6 × BALB/c) F_1 mice. Aged mice were found to have depressed T-cell and B-cell responses but increased ADCC activity. Weekly injections of bestatin over a period of 6 months resulted in varying effects depending on the dose administered. Small doses (10 μg per injection) were more effective in restoring humoral responses to SRBC rather than delayed-type hypersensitivity reactions, whereas large doses (100 μg per injection) acted in the opposite way. Macrophage activation was only obtained after the administration of the high doses of bestatin. Continuous treatment with bestatin did not prevent the appearance of suppressor cells induced by ageing. It led to a significant reduction of ADCC activity in aged animals near to the base line value of young animals. Animals were examined for the presence of spontaneous tumours from the end of the treatment until the age of 28 months. A significant reduction of spontaneous tumour incidence was observed in mice given repeated injections of 100 μg bestatin when compared to untreated aged mice and to mice given the low doses of bestatin.

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INTRODUCTION

Alteration of the immune system in the ageing process is thought to be responsible either directly or indirectly for some 'diseases of ageing' such as increase of autoimmune manifestations, incidence of infections and neoplasias (Peterson & Makinodan 1972; Good & Yunis 1974; Teller 1972; Smith, Walford & Mickey, 1973). In previous work (Bruley-Rosset, Florentin, Kiger, Davigny & Mathé, 1978), we have confirmed that this alteration affects mainly the T-cell compartment and that it could be related to thymus involution which occurs early in life. B-cell responses were affected to a lesser degree (Makinodan, Perkins & Chen, 1971) whereas macrophage functions remained unchanged (Perkins, 1971). Delay, reversal or prevention of the decline in normal immune functions may retard the onset or lessen the severity of the 'diseases of ageing'.

The following experiments deal with an assay for restoring the immune capacity of aged immunodepressed mice by a chronic treatment with bestatin, a new chemically defined immunostimulant. Isolated by Umezawa *et al.* (Ishizuka, Aoyagi & Takeuchi, 1976a; Umezawa, Aoyagi, Suda, Hamada & Takeuchi, 1976b), this antibiotic was shown to enhance both humoral and cell-mediated immune functions (Umezawa *et al.*, 1976a, b) and to display an anti-tumour activity (Umezawa, 1978). In our hands, when injected to young adult mice, bestatin was able to activate macrophages, to potentiate humoral responses to thy-

mus-dependent and thymus-independent antigens and to increase antibody-dependent cellular cytotoxicity activity of spleen cells (Florentin, Kiger, Bruley-Rosset, Schulz & Mathé, 1978).

In the present work, 16 month old mice were given weekly injections of bestatin over a period of 6 months, at the end of which they were submitted to a variety of immunological tests in order to detect the effects of the treatment on immune function of T and B lymphocytes, macrophages and K cells. Mortality of the animals and spontaneous tumour incidence were recorded after treatment ceased.

MATERIALS AND METHODS

Mice

Two- to three-month-old and sixteen-month-old (C57Bl/6 × BALB/c) F_1 mice (Centre d'elevage du CNRS, Orléans la Source, France) were used. Mice were housed in a conventional animal facility until the age of 28 months.

Schedule of bestatin administration

Bestatin, [(2S,3R)-3-amino-2-hydroxyl-4-phenylbutanoyl]-L-leucine is a specific inhibitor of aminopeptidase B and leucine aminopeptidase. It was isolated from a culture filtrate of *Streptomyces olivoreticuli* (Umezawa, Aoyagi, Suda, Hamada & Takeuchi, 1976b). Its chemical synthesis was performed by Suda, Takita, Aoyagi & Umezawa (1976).

Bestatin was kindly provided by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. It was dissoved in sterile saline and was administered weekly to 16-month-old mice by the intraperitoneal route at a dose of 10 or 100 μg per injection, over a period of 6 months. The animals were submitted to a variety of immunological tests 14 days after the last injection.

Assay for macrophage-mediated cytostasis

The *in vitro* cytostatic activity of peritoneal macrophages was determined using the tritiated thymidine (^{3}H-TdR) incorporation inhibition test described in detail previously (Florentin *et al.*, 1978). Briefly, 5×10^4 L1210 leukaemic cells in 0·2 ml RPMI 1640 culture medium were cultivated on macrophage monolayers in the wells of a culture microplate; the effector to target cell ratio was 10:1. After 18 h of incubation at 37°, cultures were pulsed with 1 μCi ^{3}H-TdR (20 Ci/mmol; CEA France) for 6 h. Tumour cells were collected on filters using a multiple harvesting device and counted for ^{3}H-TdR incorporation in a liquid scintillation spectrometer.

Results were expressed as mean c.p.m. ± standard error of twelve cultures and as percentage inhibition of tumour cell proliferation calculated by the formula:

$$\%\text{inhibition} = \frac{\text{c.p.m.}_c - \text{c.p.m.}_t}{\text{c.p.m.}_c} \times 100$$

where c.p.m._c = mean c.p.m. in cultures containing macrophages from untreated control mice and c.p.m._t in culture containing macrophages from treated mice.

Spleen cell responses to mitogens

Unfractionated spleen cells and cells fractionated by nylon wool filtration according to the method of Julius, Simpson & Herzenberg (1973) were cultivated in microplates in the presence of an optimal concentration of phytohaemagglutinin (PHA; Wellcome Laboratories, 1:500 dilution of the 5 ml reconstituted stock solution), concanavalin A (Con A; Pharmacia, 5 μg/ml), dextran sulphate (DS; Difco; 100 μg/ml), lipopolysaccharide (LPS; Difco; 100 μg/ml). Cultures were set up in triplicate, each well containing 5×10^5 cells in a volume of 0·2 ml of RPMI 1640 culture medium supplemented with 5% heat-inactivated mule serum and with antibiotics. After 48 h of incubation at 37° cultures were pulsed with 1 μCi ^{3}H-TdR per well and cells were collected 6 h later.

For detection of suppressor cells, 5×10^5 spleen cells from 2 month old normal mice were cultivated in the presence of $2{\cdot}5 \times 10^5$ unfractionated, nylon non-adherent or plastic-adherent spleen cells from bestatin-treated aged mice and stimulated with mitogens as described above.

Antibody response

Mice were immunized i.p. with 10^8 sheep red blood cells. Four days later IgM plaque-forming cells (PFC) were enumerated in the spleen by the method of Cunningham & Szenberg, 1968.

Delayed-type hypersensitivity reaction

Mice were sensitized by application of oxazolone solution to the abdomen skin. The reaction was elicited 7 days later by a second application of the sensitizing agent on both sides of each ear. Ear thickness was measured just before and 24 h after the challenge.

Antibody-dependent cell-mediated cytotoxicity (ADCC)

Various numbers of spleen cells were incubated for 18

h at 37° with 10^4 ^{51}Cr-labelled chick red blood cells (CRBC) into the wells of a microplate and in the presence of a 1:20,000 final dilution of rabbit anti-CRBC serum. The effector to target cell ratios varied from 100:1 to 12:1. The supernatants were collected and the amount of ^{51}Cr released was measured in a gamma counter. Cultures were done in triplicate and the percentage of specific lysis was calculated by the formula:

$$\%\text{lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

where the maximal release was obtained by hypotonic lysis of CRBC and spontaneous release by the mean lysis in cultures containing target and effector cells without antiserum.

Results were also expressed in terms of lytic units (LU) with one LU defined as the numbers of spleen cells required to lyse 60% of the target cells. The number of LU per spleen was calculated.

RESULTS

Mice given weekly injections of bestatin were tested at the end of a treatment of 6 months for their immunological reactivity which was compared to that of untreated animals of the same age (22 months) and of untreated 2 month old mice.

Effect of bestatin treatment on macrophage activation

As shown in Table 1, repeated injections of 10 μg of bestatin were ineffective in rendering peritoneal macrophages cytostatic for tumour cells. In contrast, when given at the dose of 100 μg per injection bestatin strongly activated macrophages which caused a 83% reduction of ^{3}H-TdR incorporation into tumour cells.

Effect of bestatin treatment on lymphocyte responsiveness to mitogens

Results presented in Table 2 show that the proliferative response of spleen cells to T-cell mitogens (PHA and Con A) was markedly depressed in 22 month old mice when compared to the response of spleen cells from 2 month old mice ($P<0.01$). Both unfractionated spleen cells and nylon non-adherent cells (T-cell enriched population) exhibited this defective response. The response to B-cell mitogens (DS and LPS) was less altered by ageing. Bestatin treatment induced a slight but not significant augmentation of the spleen-cell response to PHA and Con A. In contrast, the response to DS was increased above the normal value. A restorative effect on the response to LPS was detected only after repeated injections of 10 μg of bestatin.

Effect of bestatin treatment on suppressor cell activity

As shown in Table 3, the response of normal spleen cells from 2 month old mice to T-cell mitogens, and to B-cell mitogens, was significantly ($P<0.01$) depressed by the addition of either unfractionated or nylon non-adherent spleen cells from 22 month old mice. In

Table 1. Effect of bestatin administration to aged mice on macrophage activation. *In vitro* cytostatic activity of peritoneal macrophages for lymphoid leukaemic cells

		Dose of bestatin per injection*	
	Untreated mice	10 μg	100 μg
Mean c.p.m. ± SE	19,687 ± 4,898	18,937 ± 4,048	3,416 ± 1,396
Inhibition of tumour† cell proliferation(%)	—	4	83
Significance		$P>0.05$	$P<0.001$

* Mice were given 10 or 100 μg of bestatin weekly from the age of 16 months, over a period of 6 months.

† $$100 \times \frac{\text{c.p.m. in cultures of untreated macrophages} - \text{c.p.m in cultures of bestatin-treated macrophages}}{\text{c.p.m. in cultures of untreated macrophages}}$$

Table 2. Effect of bestatin administration to aged mice on spleen-cell mitogen responsiveness *in vitro*

Spleen-cell population tested	Mitogen	2 month old untreated mice	22 month old mice		
			Untreated mice	Dose of bestatin per injection	
				10 μg	100μg
Unfractionated spleen cells	—	577 ± 36	1,452 ± 263	830 ± 160	1,785 ± 204
	PHA	24,714 ± 1,599	6,802 ± 1,570*	7,865 ± 113*	9,064 ± 862*
	Con A	22,524 ± 2,060	7,458 ± 1,310*	11,658 ± 1,593*	11,885 ± 1,022*
	DS	4,302 ± 1,373	3,577 ± 766	5,424 ± 501	6,153 ± 623
	LPS	10,244 ± 1,701	6,518 ± 366*	11,091 ± 922	7,825 ± 1050*
Nylon non-adherent spleen cells	—	152 ± 9	452 ± 52	645 ± 122	530 ± 6
	PHA	25,099 ± 1,660	9,136 ± 487*	12,490 ± 960*	13,528 ± 621*
	Con A	26,101 ± 4,459	7,686 ± 1,975*	9,709 ± 1,091*	8,648 ± 868*
	DS	407 ± 65	839 ± 169	1,471 ± 100	1,118 ± 102
	LPS	5,765 ± 161	3,788 ± 310*	2,941 ± 522*	2,064 ± 450*

* $P < 0{\cdot}01$. The response of aged untreated or bestatin-treated animals is depressed compared to the response of young animals.

Results are given as mean c.p.m. ± SE.

Table 3. Mitogen responses of spleen cells from 2 month old mice co-cultivated with spleen cells from young and aged mice treated or not with bestatin

Cell population co-cultivated* with normal spleen cells	Mitogen	Untreated young mice	Untreated aged mice	Dose of bestatin per injection	
				10 μg	100 μg
Unfractionated spleen cells	—	1,512 ± 296*	3,343 ± 563	3,230 ± 176	3,185 ± 353
	PHA	32,688 ± 2,186	25,665 ± 401†	20,193 ± 1,190†	20,065 ± 1,299†
	Con A	40,473 ± 10,409	16,530 ± 2,250†	20,598 ± 1,730†	21,993 ± 2,921†
	DS	17,698 ± 4,783	7,877 ± 1,702†	14,224 ± 401	11,677 ± 1,240†
	LPS	18,380 ± 1,495	13,104 ± 2,293†	18,841 ± 123	12,762 ± 1,146†
Nylon non-adherent spleen cells	—	1,077 ± 291	2,329 ± 485	3,867 ± 37	3,364 ± 217
	PHA	33,287 ± 3,177	23,878 ± 1,719*	34,974 ± 3,114	21,623 ± 3,669†
	Con A	33,598 ± 7,339	17,970 ± 1,411†	24,199 ± 2,667†	20,437 ± 4,134†
	Ds	10,303 ± 845	10,325 ± 1,457	11,234 ± 2,409	11,956 ± 286
	LPS	13,959 ± 3,243	12,851 ± 1,368	11,454 ± 62	10,440 ± 1,238
Plastic-adherent spleen cells	—	404 ± 84	1,384 ± 185	3,134 ± 340	2,665 ± 436
	PHA	17,975 ± 2,460	18,459 ± 430	26,687 ± 2,292‡	24,844 ± 1,979
	Con A	25,254 ± 2,048	29,390 ± 501	44,209 ± 269‡	32,363 ± 2,298‡
	DS	4,429 ± 487	7,544 ± 600‡	8,184 ± 652‡	13,356 ± 2,352‡
	LPS	7,315 ± 1,502	8,348 ± 507	11,773 ± 2,220‡	11,047 ± 2,719‡

* 5×10^5 spleen cells from 2 month old mice were cultivated with $2{\cdot}5 \times 10^5$ spleen cells from either untreated young mice, untreated aged mice, or bestatin-treated aged mice. Mean c.p.m. ± SE.

† $P < 0{\cdot}01$. The response of aged untreated or bestatin-treated animals is depressed compared to the response of young animals.

‡ $P < 0{\cdot}01$. The response of aged untreated or bestatin-treated animals is stimulated compared to the response of young animals.

contrast, plastic-adherent spleen cells from aged mice were ineffective in decreasing the response of normal spleen cells from young adult mice. These results demonstrate the presence of non-specific suppressor cells in the spleen of aged mice which may explain the defective response of spleen cells to mitogens.

Spleen cells from bestatin-treated mice always inhibited the proliferative response to PHA and Con A of normal spleen cells from 2 month old mice. Therefore, the treatment with bestatin was ineffective in eliminating suppressor cells induced by ageing.

Effect of bestatin treatment on antibody response to SRBC

The antibody response to SRBC has been shown to decline with advancing age (Bruley-Rosset *et al.*, 1978). Weekly injections of 10 μg of bestatin resulted in a 2·4-fold increase in the number of PFC per spleen when compared to the PFC response of untreated aged mice (Table 4). In contrast, repeated administration of 100 μg of bestatin failed to improve the antibody response of aged mice.

Effect of bestatin treatment on delayed-type hypersensitivity

The intensity of the delayed-type hypersensitivity reaction to oxazolone, measured by the augmentation of ear thickness 24 h after challenge, was markedly reduced in 22 month old mice compared to the response of 2 month old animals (Table 5). A complete restoration of the response was observed in aged mice given repeated injections of 100 μg of bestatin. In contrast, 10 μg multiple injections resulted in a further decrease of delayed-type hypersenstivity.

Table 4. Effect of bestatin administration to aged mice on the plaque-forming cell (PFC) response to sheep red blood cells

		Treated mice	
		Dose of bestatin per injection*	
	Untreated mice	10 μg	100 μg
Mean number of PFC/spleen ± SE†	26,800 ± 17200	64,200 ± 22,600	36,000 ± 28,600
Significance		$0{\cdot}02 < P < 0{\cdot}01$	$P > 0{\cdot}05$

* Mice were given 10 or 100 μg of bestatin weekly from the age of 16 months over a period of 6 months.
† Ten animals per group.

Table 5. Effect of bestatin administration to aged mice on delayed-type hypersenstivity to oxazolone

	Untreated 2 month old mice	Untreated 22 month old mice	Dose of bestatin per injection*	
			10 μg	100 μg
Mean ear thickness increment ± SE† (1/100e mm)	7·67 ± 1·76	3·71 ± 0·95	1·75 ± 0·38	9·38 ± 1·84
Significance		$P < 0{\cdot}01$	$P > 0{\cdot}05$	$0{\cdot}05 < P < 0{\cdot}02$

* Mice were given 10 or 100 μg of bestatin weekly from the age of 16 months, over a period of 6 months.
† Five animals per group.

Table 6. Effect of bestatin administration to aged mice on antibody-dependent cellular cytotoxicity against ^{51}Cr-labelled CRBC

Mice	Treatment	Percentage specific lysis (±SE) for different effector:target cell ratio 100:1	50:1	25:1	LU_{60}† per culture ($\times 10^{-4}$)	Number of LU_{60} per spleen
Young mice	—	66±1·7	66±1·1	49±1·7	38	184
Aged mice	—	86±0·5*	78±2·9*	66±1·7*	21	561
Aged mice	10 μg bestatin	70±2·3*	71±2·3*	58±1·7*	29	324
Aged mice	100 μg bestatin	65±1·7	57±1·1	55±1·7*	65	218

* Significantly higher than the values observed in the group of young mice, $P<0·001$.
† Number of spleen cells required to lyse 60% of 10^4 ^{51}Cr-labelled CRBC.

Effect of bestatin treatment on ADCC

In Table 6, the values of LU_{60} per culture and the total number of LU_{60} per spleen were recorded. We can notice that a higher number of effector cells were required to obtained a 60% specific lysis of 10^4 target cells in young mice than in aged mice, indicating that spleen cells from aged mice were 1·8-fold more cytotoxic than those of young mice ($P<0·001$). As the total number of spleen cells was higher in aged mice, the number of LU_{60} per spleen showed a three-fold increased ADCC activity compared to that of young mice. The treatment with 10 μg and even more with 100 μg of bestatin resulted in an increase of the LU_{60} values per culture. This indicates a decrease of ADCC activity of spleen cells of aged animals as a consequence of continuous bestatin treatment. When considering the number of lytic units per spleen, however, the 10 μg bestatin-treated mice exhibited a 1·7-fold higher ADCC activity in comparison to young mice whereas the level of cytotoxicity in the 100 μg bestatin-treated group was lowered to the values observed in the group of young mice.

Effect of bestatin treatment on spontaneous tumour incidence and on life span

The mortality of the animals was recorded during and after the treatment with bestatin (Fig. 1). Chronic administration of bestatin does not seem to be toxic since only one out of twenty mice died during the treatment. At 28 months of age (6 months after the end of the treatment), 50% of untreated mice were still alive against 57% in the group of animals treated with

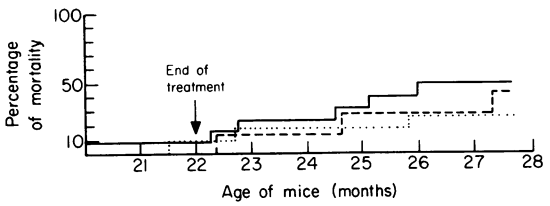

Figure 1. Percentage mortality related to ageing in mice either untreated (solid line) or treated repeatedly with bestatin at the doses of 10 μg (dashed line) or 100 μg (dotted line).

10 μg of bestatin and 73% in the group of mice given 100 μg bestatin (difference not significant). At that time, the animals were killed and examined for the presence of spontaneous tumours. The animals that died before the age of 28 months were also checked for the presence of tumours. In the control group, 36% of the mice were found to bear a malignant tumour whereas tumours were detected in only 18% ($P>0·05$) and 5% ($P=0·05$) of the mice treated with 10 and 100 μg of bestatin respectively. Histological examination revealed that 90% of these tumours were undifferentiated or poorly differentiated lymphosarcomas, and that they were located in spleen, liver and abdominal lymph nodes.

DISCUSSION

Recent observations by Umezawa *et al.* (1976) and Florentin *et al.* (1978) clearly demonstrate the immunostimulatory properties of bestatin on immune responses of immunocompetent young adult mice.

This prompted us to investigate whether this new chemically defined immunostimulant could also restore to normal the impaired immune functions of aged mice.

First, we have analysed the immunological changes induced by ageing in the particular strain of (C57Bl/6 × BALB/c)F_1 hybrid mice that we submitted to bestatin treatment. Two- to three-month-old mice were used as adult immunocompetent mice. It has been shown by several investigators that among immune parameters studied in the present paper, the ADCC activity and the lymphoproliferative response to T mitogens can be considered as fully developed in even 2 month old mice (Pollack & Kraft, 1977; Kay, 1978). With respect to antibody response to various antigens we are aware that the response is maximal only in 3–6 month old mice (Doria, Dagostovo & Poretti, 1975); The difference between 2 and 3 month old mice in respect to this antibody response is negligible. In this study we confirmed that T-cell functions are strongly affected by ageing. Indeed, a depression of delayed-type hypersensitivity to oxazolone, of antibody formation against a thymus-dependent antigen (SRBC) and of spleen-cell responsiveness to PHA and Con A was observed in 22 month old mice as compared to 2–3 month old animals. These effects may be related to the presence of non-specific suppressor cells in the spleen of aged animals. In the present study, such cells were detected by their capacity to inhibit the T-cell mitogen response of spleen cells from young adult mice. They were recovered in the nylon non-adherent cell population and not in the plastic-adherent one which suggests that they belonged to the T-cell lineage. Suppression of a variety of immune functions by admixture of young and old lymphoid cells has already been reported both *in vito* and *in vitro* (Peter, 1973; Halsall & Makinodan 1972; Makinodan, Algright, Good, Peter & Heidrick, 1976; Segre & Segre, 1976; Goidl, Innes & Weksler, 1976). The decline in T-cell immune responses has been also explained by a decrease in thymic hormone level (Goldstein, Hooper, Schulof, Cohen, Thurman, McDaniel, White & Dardenne, 1974), and/or a reduction in helper T-cell activity (Goidl *et al.*, 1976; Friedman & Globerson, 1978). The responses to thymus-independent antigens and to B-cell mitogens are also impaired by ageing but to a lesser extent than thymus-dependent immune reactions (Makinodan *et al.*, 1971; Gerbase-De Lima, Wilkinson & Smith, 1974; Kishimoto, Takahama & Mizumachi, 1976; Gullard, Bastena & Waters, 1977; Meredith & Walford, 1977). In this study, we observed a depression of spleen cell response to DS and LPS but we failed to demonstrate that suppressor cells were responsible for this defective response. Macrophage functions do not seem to be altered by ageing (Perkins & Makinodan, 1971; Heidrick & Makinodan, 1973).

In the present work, we reported that the immunological reactivity of 22 month old mice could be modified after a long term administration of bestatin, the effects of the treatment depending on the dose of bestatin injected. Repeated administration of small doses of bestatin (10 μg per weekly injection) over a period of 6 months, was effective in restoring the humoral response against SRBC but it resulted in a further decrease of delayed-type hypersensitivity to oxazolone. Larger doses (100 μg per injection) acted in the opposite way: a complete restoration of delayed-type hypersensitivity was observed whereas the PFC response to SRBC was not signficantly affected. These results are in accordance with previous observations by Umezawa *et al.* (1976a, b) who reported that a large dose of bestatin (> 100 μg) when injected in young adult mice, potentiated the humoral response to SRBC whereas a low dose (< 10 μg) exhibited greater activity in enhancing delayed-type hypersensitivity to the same antigen.

Bestatin treatment was ineffective in restoring the defective response of spleen cells to PHA, Con A and LPS. It also failed to prevent the appearance of suppressor cells or to diminish their activity. This contrasts with our previous observations on the effects of a similar immunorestorative treatment with levamisole (Bruley-Rosset *et al.*, 1979). The long term administration of this agent led to a restoration of delayed-type hypersensitivity and humoral responses to thymus-dependent antigens and of spleen cell responses to T-cell mitogens. Concomitantly, we observed the absence of non-specific suppressor cells in the spleen of levamisole-treated mice.

Macrophages from mice given repeated injections of 100 μg bestatin were highly cytostatic for tumour cells. No macrophage activation could be detected after treatment with the low dose of bestatin. This suggests that T cells were implicated in the macrophage activating process.

Surprisingly, ADCC activity of spleen cells against antibody-coated CRBC was markedly augmented in 22 month old mice when compared to 2 month old animals. Bestatin treatment resulted in a return of ADCC activity close to the base-line value of young animals. The high dose of bestatin was more effective than the low dose in reducing spleen cell lytic activity.

Levamisole was shown to exert a similar normalizing effect on the ADCC activity in aged mice (Bruley-Rosset *et al.*, 1979).

The immune functional changes which occur during ageing are likely to contribute to the pathogenesis of various diseases which show a peak incidence in late life and particularly to the development of neoplastic disease (Burnet, 1970; Smith *et al.*, 1973).

In the present study, we observed that spontaneous tumour incidence was significantly lower in mice given repeated injections of 100 μg bestatin than in untreated mice or mice treated with the low dose of bestatin. This effect was still detected 6 months after the onset of the immunorestorative treatment which also increased (but not significantly, due to the small number of mice per group) the lifespan of the animals. Similar observations have been done in levamisole-treated aged mice. In contrast, repeated administration of 1 mg BCG was shown to reduce the mean survival time of the animals (Bruley-Rosset *et al.*, 1978).

From all these results, it is tempting to speculate about the immunological mechanism(s) which should be stimulated in order to increase the life expectancy and to reduce the frequency of neoplasia in aged animals. Activated macrophages are known to play an important role in anti-tumor immunity. Repeated administration of 100 μg bestatin, 75 μg levamisole or 1 mg BCG in aged animals (Bruley-Rosset *et al.*, 1978) induced macrophage activation. Both the former treatments led to reduction in spontaneous tumour incidence and in prolongation of life span but the latter (BCG) had the opposite effect. Heightened ADCC activity observed in aged mice and its normalization after bestatin or levamisole treatment, but not after BCG treatment may be related to the presence of spontaneous tumour occurrence. Indeed, a correlation between high ADCC activity and tumour growth has been established in mice bearing grafted tumour (Ghaffard, Calder & Irvine, 1976). Moreover, this mechanism of cytotoxicity has been shown to play an important role in mediating the antitumoral effect of BCG in leukaemic mice (Olsson, Florentin, Kiger & Mathe, 1977). Considering the differential effects of bestatin on the immune responses in relation to the dose injected, a restoration of delayed-type hypersensitivity and modulation of ADCC activity seem to correlate more with an anti-tumour effect than does restoration of humoral immunity.

Administration of immunostimulating agents is the more attractive approach to correct immunodepression in old age. The present study demonstrates the efficacy of bestatin in restoring the impaired immune functions of ageing mice. These results are very promising in view of the absence of toxicity of this chemically well defined molecule demonstrated both in animals and humans (Umezawa *et al.*, 1978). It is therefore more suitable for chemical application than levamisole which exerts similar but often questionable immunorestorative effects (reviewed by Symoens & Rosenthal, 1977, and which is not devoid of toxicity (Werner *et al.*, 1977).

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