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Effect of anticancer agents neothramycin, aclacinomycin, FK-565 and FK-156 on the release of interleukin-2 and interleukin-1 in vitro

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Summary. Four anticancer agents, neothramycin, aclacinomycin, FK-565 and FK-156, were tested for their effects on concanavalin-A-induced interleukin-2 release from rat splenocytes in vitro. Neothramycin showed an enhancement of the release of interleukin-2, whereas aclacinomycin had no effect. FK-565 and FK-156 were found to inhibit the release of interleukin-2 under similar conditions. The inhibition was much more marked with FK-565. These drugs were also tested for their effects on the release of interleukin-1 from rat peritoneal exudate cells stimulated by lipopolysaccharide in vitro. Neothramycin and aclacinomycin did not affect the release of interleukin-1; however, both FK-565 and FK-156 resulted in its enhanced release under these conditions.

Introduction

In previous studies we have investigated the anticancer agents bleomycin, adriamycin, cyclophosphamide, vincristine and vinblastine for their effect on the release of interleukin-2 (IL-2) and interleukin-1 (IL-1) in vitro and in vivo [1–4, 20]. We now report the effects of further experimental anticancer drugs on IL-2 and IL-1 release in vitro.

Neothramycin and aclacinomycin have been reported as antibiotics with antitumour effects [8, 22]. Neothramycin is derived from cultures of *Streptomyces thioluteus*. It has been shown to be active against experimental tumours such as lymphocytic leukaemia P388, ascites sarcoma −180, hepatoma AH130, Walker carcinosarcoma 256 and mouse mammary adenocarcinoma [9]. Neothramycin has also been found to augment immune responses. When mice were injected with neothramycin at the time of immunization, IgM antibody formation and delayed-type hypersensitivity to sheep red blood cells were enhanced over a wide dose range [12]. It has been suggested that part of its antineoplastic effect may be mediated by alteration of the host's immune response [16]. Aclacinomycin is derived from cultures of *Streptomyces galilaeus*. It has antibacterial activity and antitumour activity against L-1210, P-388 and other murine tumours [19, 21]. Ishizuka et al. [11] have reported enhancement of delayed type hypersensitivity reactions and antibody formation by aclacinomycin when injected into mice before or after immunization.

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FK-156 and FK-565 are novel peptides known to be immunoactive in certain conditions [17]. FK-156 was isolated from culture filtrates of *Streptomyces olivaceogriseus* sp. nov. and *Streptomyces violaceus* [7]. FK-565 is a synthetic analogue of FK-156. Both these peptides have been reported to enhance resistance to a variety of infections and are effective against experimental tumours such as P388, a mouse lymphocytic leukaemia of DBA/2 origin [13, 17].

T cells and macrophages are considered to play an important role in the defence against cancer [10, 20]. We have, therefore, screened these anticancer agents, which have already been shown to have an immunomodulatory potential, for their effects on two of the cytokines most closely linked with T cell and macrophage function, that is, IL-2 and IL-1. These studies show IL-2 production to be enhanced by neothramycin, whereas IL-1 production is enhanced by the FK peptides, which also inhibit the production of IL-2. Aclacinomycin, however, was shown to be ineffective in studies on both the cytokines investigated.

Materials and methods

Animals. Male Lewis rats were purchased from Bantin and Kingman (Hull, UK). They were fed FFG(M) diet and water ad libitum. They weighed 250 g at the time of the experiment.

Drugs. Neothramycin and aclacinomycin were kind gifts from Dr M Ishizuka of the Institute of Microbial Chemistry, Microbial Chemistry Research Foundation, Tokyo, Japan. FK-156 and FK-565 were kindly provided by the Fujisawa Pharmaceutical Company Ltd, Osaka, Japan. All the drugs were dissolved in complete medium immediately before use. Human recombinant interleukin-2 was the kind gift of Hoffmann La Roche (New Jersey, USA). Purified human interleukin-1 was purchased from Koch-Light (UK).

Spleen cell suspension. Rats were killed and their spleens removed aseptically, chopped over a fine wire mesh, gently teased and debris removed. Erythrocytes were haemolysed by hyposmotic shock and remaining cells were washed three times in Hank's balanced salt solution, (HBSS, Wellcome). Cells were finally suspended in RPMI-1640 (Flow Labs, Irvine, Scotland) supplemented with 50 IU/ml penicillin (Flow), 50 μg/ml streptomycin

(Flow), 2.5 mM sodium pyruvate (Gibco, Uxbridge, UK), 1 mM L-glutamine (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma, Dorset, UK) and 10% heat-inactivated fetal calf serum (Seralab, Sussex, UK). This formula is referred to throughout this work as complete medium. Cell viability was determined by trypan blue exclusion.

Production of interleukin-2. The method described by Gillis et al. was used [6]. Briefly, 1×10^6 splenocytes/ml were incubated with concanavalin A (ConA) (Pharmacia, Bucks), 0.5 μg/ml at 37° C with or without different concentrations of the drug in six-well culture plates (Nunc, Denmark). After 24 h, supernatants were dialysed for 24 h at 4° C (two changes) against phosphate-buffered saline (Oxoid) to remove the drug before passing through a Millipore filter and storage at −20° C.

Assay for IL-2 activity. CTLL-16, an IL-2-dependent cell line, was used as the target cell population in the IL-2 microassay. The cells were the kind gifts of Dr R. Lelchuck (Wellcome Laboratories). Cells were maintained in long-term proliferative cultures in complete medium, supplemented with 25% IL-2-rich-supernatant. The assay cells were washed free of growth medium and resuspended in complete medium at a concentration of 1×10^5 cells/ml. 100-μl aliquots of cell suspension were placed into 96-well microplates (Nunc, Denmark) followed by serial dilutions of test sample (100 μl) to be assayed. Microplates were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. After 20 h 1 μC_i [^{3}H]thymidine (2C_i/mmol, Amersham International, UK) was added to each culture before incubation for a further 4 h. The cultures were harvested onto Whatman grade GF/A glass-fibre discs. The radioactivity in the filter discs was counted in a Pakard Tri Carb scintillation counter (model 2405).

Production of IL-1. The method used for IL-1 production was based on that described by Thomson et al. [23] with some modifications. In brief, peritoneal exudate cells were obtained four days after i.p. injection of 5 ml sterile paraffin oil into rats. The peritoneal cavities were lavaged with HBSS. The cells were washed three times in HBSS and re suspended in complete medium to 3×10^6 viable cells/ml. 3-ml aliquots of this peritoneal exudate cell suspension were left to adhere to six-well plastic culture plates (Nunc) for 3 h at 37° C. Non-adherent cells were removed by washing three times with complete medium, then 2 ml complete medium, containing 1 μg *Escherichia coli* lipopolysaccharide B_8 (Difco, Surrey) and various concentrations of the drug, were added to the adherent cells (2.3×10^6/well). The cultures were incubated at 37° C for 24 h. The supernatants were clarified by centrifugation and dialysed for 24 h at 4° C (two changes) against phosphate-buffered saline. They were then passed through a Millipore filter and stored at −20°C.

Assays for IL-1 activity. D10.G4.1 cells were used as the target cells in the assay for IL-1. These cells are a conalbumin-specific AKR T-cell clone, isolated and characterised by Kaye et al. [14] and were the kind gift of Professor M. Feldmann of Charing Cross Hospital, London. The cells were maintained by weekly passage with irradiated syngeneic spleen cells and conalbumin (Sigma). The assay method was described by Matsushima et al. [15]. 100-μl aliquots of cell suspension in complete medium containing 2×10^4 cells and 5 μg/ml ConA were placed in 96-well microculture plates followed by various dilutions of the test supernatant. Controls included cells plus medium alone and cells plus purified human IL-1. The plates were incubated at 37° C for 48 h with a 4-h pulse with 1 μCi [^{3}H]thymidine (5 Ci/mmol). As the D10.G4.1 cells respond also to IL-2, control IL-1 supernatants were tested for any IL-2 activity against CTLL-16 cells [14]. No IL-2 activity was detected.

Statistical analysis. Results are expressed as mean [^{3}H]thymidine incorporation (cpm) ± SD of triplicate samples. The significance of differences between the means was evaluated by the analysis of variances and by Student's *t*-test.

Results

Effect on the release of interleukin-2

Neothramycin. Neothramycin was found to enhance the release of interleukin-2 at the doses of 1 ng/ml and 10 ng/ml. The drug was found to be directly cytotoxic to rat splenocytes at the dose of 100 ng/ml (as determined by trypan blue exclusion). As shown in Fig. 1, 1 ng/ml neo-

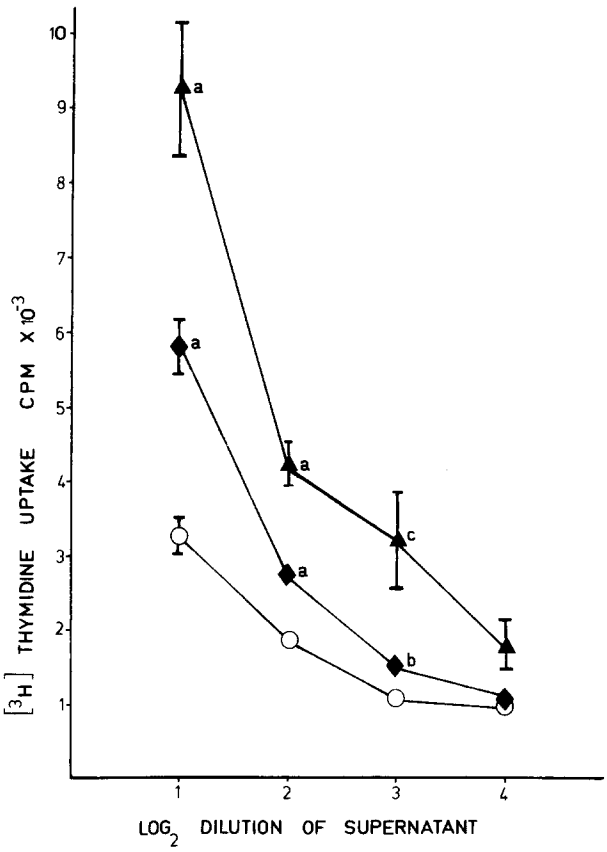

Fig. 1. Effect of neothramycin on the release of interleukin-2 by rat splenocytes in vitro. 1×10^6 splenocytes/ml were incubated with ConA (0.5 μg/ml) and 0 (○——○), 1 ng/ml (▲——▲) or 10 ng/ml (◆——◆) neothramycin. Supernatants were harvested after 24 h, dialysed to remove the drug and then assayed for IL-2 activity against the IL-2-dependent cell line (CTLL-16). The results are given as means [^{3}H]thymidine incorporation (cpm) ± SD. The negative control of cell line and medium alone gave 1086±92 cpm. *a*, $P < 0.001$; *b*, $P < 0.005$; *c*, $P < 0.01$ as compared with cultures without neothramycin

Table 1. Effect of aclacinomycin on the release of interleukin-2 by rat splenocytes in vitro[a]

Dose (ng/ml)	[^{3}H]Thymidine incorporation (cpm); dilutions			
	1/2	1/4	1/8	1/16
0	8368.5 ± 3247.7	4642.0 ± 301.6	2724 ± 153.8	1435.8 ± 225.5
1	7048.5 ± 351.2	5669.3 ± 419.6	3743.1 ± 1013.4	2174.3 ± 285.2
10	5733 ± 235.9	3881 ± 385.3	1868.6 ± 136	1409.6 ± 276
100	7374.5 ± 177.4	4645.1 ± 191.1	3235 ± 109.4	2055 ± 340.9

[a] 1×10^6 splenocytes/ml were incubated with ConA (0.5 μg/ml) and 0, 1, 10 and 100 ng/ml aclacinomycin. Supernatants were harvested after 24 h, dialysed to remove the drug and then assayed for IL-2 activity against an IL-2-dependent cell line (CTLL-16). The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of the cell line and medium alone gave 877 ± 256 cpm

thramycin increased IL-2 activity in the supernatant by 184% over control levels when both supernatants were compared at the 1:2 dilutions in IL-2 microassay ($P < 0.001$). This increase was less marked (79% higher activity as compared with controls, $P < 0.001$) at the dose of 10 ng/ml. Similar effects were seen in three out of five animals.

Aclacinomycin. Aclacinomycin did not affect the release of interleukin-2 in any of the three animals tested over a non-cytotoxic dose range of 1–100 ng/ml (Table 1, representative of three separate experiments). 1 μg/ml aclacinomycin was found to be directly cytotoxic to rat splenocytes (as determined by trypan blue exclusion).

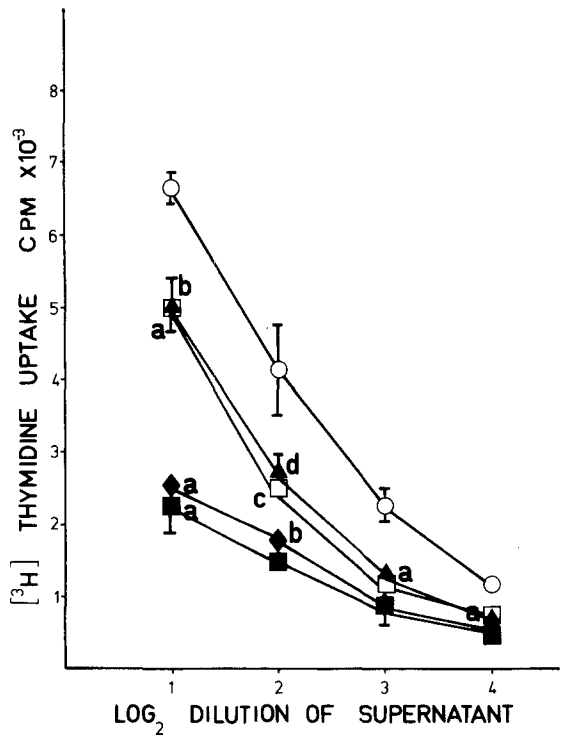

Fig. 2. Effect of FK-565 on the release of interleukin-2 by rat splenocytes in vitro. 1×10^6 splenocytes/ml were incubated with ConA (0.5 μg/ml) and 0 (○——○), 1 ng (▲——▲), 10 ng/ml (□——□), 100 ng/ml (◆——◆) and 1 μg/ml (■——■) FK-565. Supernatants were harvested after 24 h, dialysed to remove the drug and then assayed for IL-2 activity against the IL-2-dependent cell line (CTLL-16). The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. The negative control of cell line and medium alone gave 687 ± 23 cpm. *a*, $P < 0.001$; *b*, $P \pm 0.005$; *c*, $P < 0.02$; *d*, $P < 0.05$ as compared with cultures without FK-565

FK-565. FK-565 resulted in an inhibition of interleukin-2 activity in the non-cytotoxic dose range of 1 ng/ml to 1 μg/ml. Test supernatants from 1 ng/ml FK-565-treated cultures, when compared with the untreated controls at 1:2 dilution, showed 25% less IL-2 activity ($P < 0.005$) (Fig. 2, representative of three separate experiments). This inhibition increased to 65% at the dose of 1 μg/ml ($P < 0.001$). The drug was only found to be directly cytotoxic to rat splenocytes at 10 μg/ml.

FK-156. FK-156 was tested over a non-cytotoxic dose range of 1 ng/ml to 10 μg/ml. It resulted in a slight inhibition of IL-2 activity. When test supernatants were compared at 1:2 dilution, cultures treated with 1 ng/ml FK-156 resulted in 9% lower IL-2 activity ($P < 0.05$) as compared with untreated controls). The inhibition was less marked at 10 ng/ml (8% less activity at 1:2 dilution of the test supernatant, $P < 0.05$ compared with untreated controls) (see Table 2, representative of three separate experiments).

Effect on the release of interlenkin-1

Neothramycin and aclacinomycin. Neothramycin did not affect the release of interleukin-1 significantly in any of the three animals treated over a dose range non-cytotoxic to rat peritoneal exudate cells (Table 3, representative of three separate experiments). Similarly, aclacinomycin did not alter the release of interleukin-1 at various non-cytotoxic doses tested (Table 4, representative of three separate experiments).

FK-565. FK-565 at doses of 1 ng/ml, 10 ng/ml and 100 ng/ml resulted in a significantly higher IL-1 activity (Fig. 3, representative of three separate experiments) as compared with controls. At 1:8 dilution of the supernatant, a *19%* higher activity was seen, as compared with untreated controls ($P < 0.05$). This activity increased to *35%* higher at 10 ng/ml ($P < 0.005$), whereas 100 ng/ml resulted in a *24%* higher IL-1 activity ($P < 0.005$ as compared with untreated controls). At 1:16 dilution of the supernatant, 10 ng/ml produced *29%* higher IL-1 activity ($P < 0.005$ as compared with controls). There were a num-

Table 2. Effect of FK-156 on the release of interleukin-2 from rat splenocytes in vitro[a]

Dose (ng/ml)	[^{3}H]Thymidine incorporation (cpm); dilutions			
	1/2	1/4	1/8	1/16
0	6372 ± 229	7477 ± 338	5503 ± 449	3227 ± 211
1	5812 ± 218[e]	5934 ± 452[c]	4452 ± 290[e]	2321 ± 96[b]
10	5869 ± 26[e]	6781 ± 166[e]	5434 ± 462	3396 ± 5511
100	6337 ± 336	7074 ± 284	5045 ± 456	2943 ± 162
1 000	6465 ± 209	6336 ± 561[e]	4513 ± 204[e]	2464 ± 201[d]
10 000	6679 ± 423	6859 ± 124	4522 ± 181[e]	2829 ± 359

[a] 1×10^6 splenocytes/ml were incubated with ConA (0.5 μg/ml) and 0, 1, 10, 100 ng/ml, 1 μg/ml and 10 μg/ml FK-156. Supernatants were harvested after 24 h, dialysed to remove the drug and then assayed for IL-2 activity against an IL-2-dependent cell line (CTLL-16). The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of the cell line and medium alone gave 529 ± 189 cpm
[b] $P < 0.005$; [c] $P < 0.01$; [d] $P < 0.02$; [e] $P < 0.05$ as compared with cultures without FK-156

Table 3. Effect of neothramycin on interleukin-1 production by rat peritoneal exudate cells in vitro[a]

Dose (ng/ml)	[^{3}H]Thymidine incorporation (cpm); dilutions							
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0	46 588 ± 3989	54 638 ± 1516	55 944 ± 6541	59 993 ± 4415	65 618 ± 6089	57 869 ± 5037	54 692 ± 4703	64 913 ± 7233
1	50 155 ± 3259	56 347 ± 4412	58 710 ± 1920	66 842 ± 2953	64 998 ± 5834	63 927 ± 1417	67 002 ± 4508	67 880 ± 2984
10	51 955 ± 769	62 500 ± 5159	65 816 ± 506	67 119 ± 3143	68 160 ± 5275	69 251 ± 1143	70 448 ± 3737	66 218 ± 4137
100	51 997 ± 2815	62 604 ± 496	67 775 ± 3267	69 462 ± 4314	62 164 ± 8653	65 639 ± 4303	65 896 ± 2439	64 961 ± 2598

[a] Adherent cells from 9×10^6 peritoneal exudate cells were incubated with 0.5 μg/ml lipopolysaccharide and 0, 1, 10 and 100 ng/ml neothramycin. Supernatants were dialysed to remove the drug, then assayed for IL-1 activity against the D10.G4.1 cell line. The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of the cell line plus medium alone gave 1067 ± 114 cpm

Table 4. Effect of aclacinomycin on interleukin-1 production by rat peritoneal exudate cells in vitro[a]

Dose (ng/ml)	[^{3}H]Thymidine incorporation (cpm); dilutions						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0	5344 ± 796	8399 ± 482	8851 ± 1306	13 771 ± 1408	10 456 ± 353	12 859 ± 1318	13 073 ± 264
1	5051 ± 184	7516 ± 905	8726 ± 946	10 080 ± 781	11 094 ± 613	11 115 ± 1247	12 080 ± 526
10	6611 ± 570	9189 ± 243	10 122 ± 571	13 158 ± 1438	13 175 ± 2584	13 404 ± 474	13 427 ± 1448
100	3549 ± 654	7251 ± 891	10 663 ± 682	13 327 ± 1479	15 460 ± 2667	12 875 ± 72	14 691 ± 1485

[a] Adherent cells from 9×10^6 peritoneal exudate cells were incubated with 0.5 μg/ml lipopolysaccharide and 0, 1, 10 and 100 ng/ml aclacinomycin, supernatants were dialysed to remove the drug, then assayed for IL-1 activity against the D10.G4.1 cell line. The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of the cell line plus medium alone gave 132 ± 25 cpm

ber of other points of significant enhancement at different test doses and supernatant dilutions. These effects were less obvious in one of the three animals tested.

FK-156. FK-156 also showed an enhancement of IL-1 release at various test doses (Fig. 4, representative of three separate experiments). At 1 ng/ml FK-156, when supernatants were compared at 1:4 dilutions, there was a 20% higher IL-1 activity in the supernatants from drug-treated cultures ($P < 0.01$). This enhancement increased to 23% at 10 ng/ml ($P < 0.001$) whereas 100 ng/ml increased IL-1 activity by 18%. When supernatants at 1:16 dilution were compared, there was a *31%*, *27%* and *21%* enhancement ($P < 0.001$, $P < 0.005$, and $P < 0.01$) at 1 ng/ml, 10 ng/ml and 100 ng/ml respectively. These effects were less obvious in one of the three animals tested.

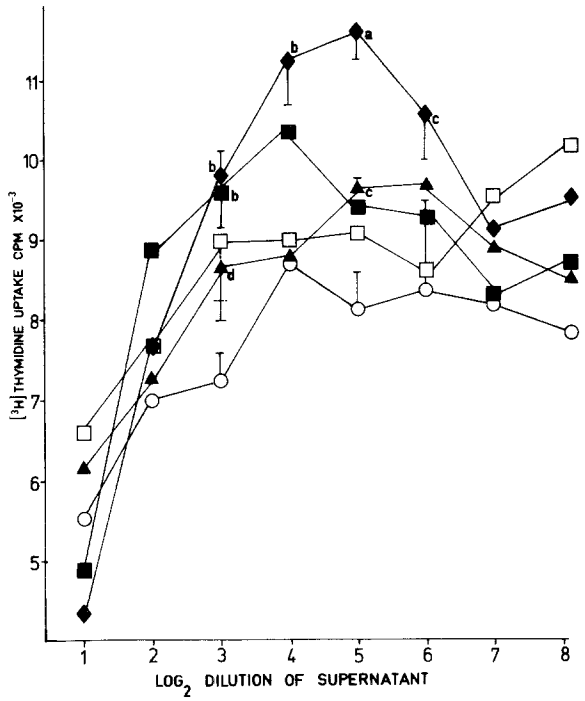

Fig. 3. Effect of FK-565 on interleukin-1 production by rat peritoneal exudate cells in vitro. Adherent cells from 9×10^6 peritoneal exudate cells were incubated with 0.5 μg/ml lipopolysaccharide and 0 (○——○), 1 ng/ml (▲——▲), 10 ng/ml (◆——◆), 100 ng/ml (□——□) or 1 μg/ml (■——■) FK-565. Supernatants were harvested after 24 h, dialysed to remove the drug, then assayed for IL-1 activity against the D10.G4.1 cell line. The results are given as mean [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of cell line plus the medium alone gave 1629 ± 161 cpm. *a*, $P < 0.001$; *b*, $P \pm 0.005$; *c*, $P < 0.02$; *d*, $P < 0.05$ as compared with cultures without FK-565

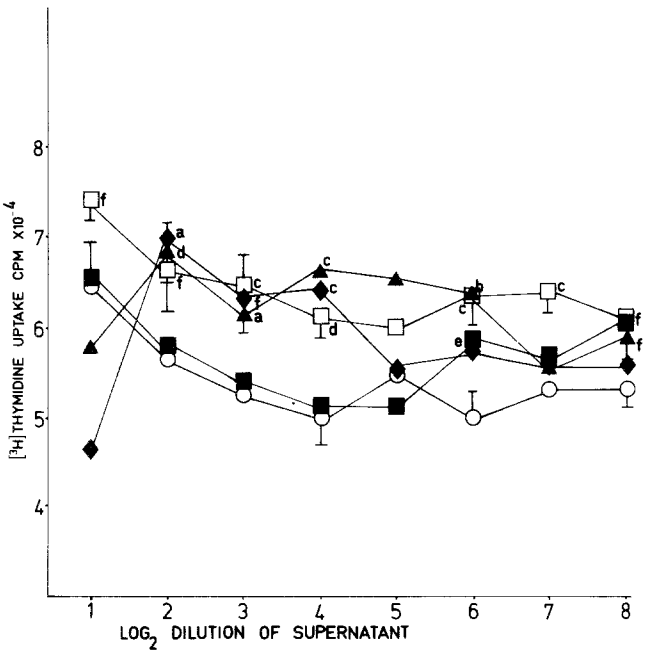

Fig. 4. Effect of FK-156 on interleukin-1 production by rat peritoneal exudate cells in vitro. Adherent cells from 9×10^6 peritoneal exudate cells were incubated with 0.5 μg/ml lipopolysaccharide and 0 (○——○), 1 ng/ml (▲——▲), 10 ng/ml (◆——◆), 100 ng/ml (□——□) or 1 μg/ml (■——■) FK-156.Supernatants were dialysed to remove the drug, then assayed for IL-1 activity against the D10.G4.1 cell line. The results are given as [^{3}H]thymidine incorporation (cpm) ± SD. Negative control of the cell line plus medium alone gave 2567 ± 838 cpm. *a*, $P < 0.001$; *b*, $P < 0.002$; *c*, $P < 0.005$; *d*, $P < 0.01$; *e*, $P < 0.02$; *f*, $P < 0.05$ as compared with cultures without FK-156

Discussion

In this study four recently developed anticancer drugs were tested for their capacity to alter the in vitro production of IL-2 and IL-1 by rat spleen cells and adherent peritoneal macrophages respectively. We have previously demonstrated an enhancement of IL-2 and IL-1 production by the antitumour antibiotics bleomycin and adriamycin [1, 2]. Enhancement of interleukin-2 activity by neothramycin could be related. Although bleomycin and adriamycin enhanced IL-1 production, this was much less than the enhancement of IL-2. The enhancement of IL-2 by neothramycin, with no effect on the release of IL-1, differs from the effect of bleomycin and adriamycin. It could be that this indicates action through a different pathway. Enhanced IL-2 release by neothramycin is consistent with the findings of Ishizuka et al. [12] that in mice injected with neothramycin at the time of immunization, IgM antibody formation and delayed-type hypersensitivity to sheep red blood cells were enhanced over a wide dose range. They also found that phagocytosis by peritoneal macrophages was stimulated following the injection of neothramycin in vivo and it was suggested that part of the immune enhancing effect of neothramycin might be mediated via an effect on cytokine production [12]. However, we could not detect any effect of neothramycin on IL-1 release in vitro. Ishizuka et al. [11] have also reported enhancement of DTH and increased antibody formation by aclacinomycin. We did not find any effect of aclacinomycin on either IL-1 or IL-2 release. This is more in accordance with the findings of Dickneite et al. [5], who demonstrated that aclacinomycin did not affect the DTH response to sheep red blood cells or the in vivo generation of cytotoxic T lymphocytes in mice. Likewise, it did not affect the survival time of rat skin allografts.

FK-156 and FK-565 have been known to be immunoactive under certain situations. Watanabe et al. [24] have shown that both FK-156 and FK-565, given parentally or orally to mice, enhanced spreading of peritoneal macrophages, phagocytosis of latex particles and intracellular killing of bacteria by peritoneal macrophages. However, in the same study, they found that the effects of these peptides were less marked on spleen macrophages and it was suggested that activation of free and fixed macrophages by FK-156 may occur by different mechanisms. Our findings of enhancement of IL-1 production by these peptides and inhibition of IL-2 formation suggest that the immune enhancing effects of these peptides could be mediated mainly by a direct action on macrophages rather than T cells. This is further supported by the findings of Watanabe et al. [24] that intraperitoneally injected FK-156 and FK-565 significantly enhanced the production of superoxide anion and phagocytosis by peritoneal macrophages of nude as well as normal mice. The more marked inhibition of IL-2 caused by FK-565 may be related to the observation that injec-

tions of FK-156 did not affect the body weight of mice, whereas this was markedly lowered in animals injected with FK-565. This suggested the superiority of FK-156 as an immunotherapeutic agent over FK-565 with respect to its safety in the treatment of cancer [24].

In conclusion, these findings confirm and extend the observations that these drugs have interesting immunomodulatory effects, which are exerted at different levels of the immune response. The immune enhancing potential of neothramycin appears to be due to an effect on T cell function, whereas FK-156 and FK-565 mediate their immune enhancing effect by direct activation of macrophages. Aclacinomycin did not seem to affect cytokine production in the rat.

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