Modulation of tumour colony growth by irradiated accessory cells

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Summary The ability of human tumour cells to form colonies in soft agar is enhanced by the presence of autologous phagocytic/adherent cells. We investigated the effect of irradiation on the ability of the adherent cells to support human tumour colony formation. Relatively low doses of irradiation significantly increased the growth enhancing ability of adherent cells in 17/19 cases. The possibility that the enhancement was mediated by inactivation of radiosensitive contaminating lymphocytes was explored. Depletion of T lymphocytes from unirradiated adherent cells by a monoclonal antibody and complement resulted in little overall change in tumour colony growth. However, elimination of only the suppressor subset (OKT8$^+$) of T lymphocytes resulted in increased colony growth relative to control values obtained with unirradiated adherent cells. In contrast, depletion of T lymphocytes from irradiated adherent cells by a pan T monoclonal antibody and complement decreased colony formation. Thus, the ability of irradiated macrophages to enhance tumour colony growth appeared to be mediated by a T lymphocyte. The effect of irradiation on isolated populations of macrophages and T lymphocytes was also examined. The enhanced ability of irradiated adherent cells to support tumor colony growth appeared to have been due to treatment of T lymphocytes alone. The results indicate that both adherent macrophages and lymphocytes may influence the growth of clonogenic human tumour cells.

The factors controlling the ability of human tumour cells to form colonies in soft agar are poorly understood. Clonogenic cells may respond to growth promoting or inhibiting substances produced by accessory cells. Selective manipulation and characterization of the different regulatory cell subsets are necessary to understand these complex interrelationships.

We have previously described the effect of phagocytic and adherent cells on the growth of human tumour colonies. We found that depletion of phagocytic macrophages from ovarian carcinoma effusions resulted in a decrease of tumour colonies (Hamburger *et al.*, 1978). In this case, tumour growth appeared to be macrophage-dependent. In an attempt to identify the macrophage subpopulations responsible for enhancing tumour growth, we depleted adherent cells of macrophages bearing surface Ia antigens and assessed the ability of the residual population to support growth (Hamburger & White, 1982). The results indicated that Ia$^-$ macrophages enhanced the growth of tumour cells in soft agar and that Ia$^+$ macrophages may have limited cell growth.

These observations were subsequently confirmed by Buick *et al.* (1980) using human malignant effusions derived from patients with a variety of adenocarcinomas and Schultz *et al.* (1980) using tumour cell lines and transplantable tumour systems. Similarly, Mantovani *et al.* (1979, 1980) have demonstrated that macrophages from patients with ovarian cancer enhance the growth of ovarian tumour cells under defined conditions.

To further investigate the nature of potentiating cells, we determined the effect of irradiation on the ability of adherent cells to support tumour colony growth. The results indicate irradiation of adherent cells increased their ability to enhance the growth of human tumour colonies. The possibility that a radiosensitive T lymphocyte may have inhibited the growth of tumour colonies was also explored.

Materials and methods

Patients

Pleural or ascitic fluids (200–4,000 ml) were obtained aseptically in heparinized (10 units ml^{-1}) vacuum bottles from patients with histologically-proven epithelial neoplasms. The presence of tumour cells in the fluid was verified by an independent pathologist. Appropriate informed consent was obtained in all cases.

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Cells

Fluid was passed through sterile gauze, centrifuged at 600 g for 10 min and the cell pellet resuspended in McCoy's 5A medium containing 10% foetal bovine serum (FBS). Cells were washed twice in this medium and counted in a haemocytometer. Viable nucleated cell counts (determined using erythrosin-B) were routinely >90%. Differential counts were performed on slides prepared with a cytocentrifuge and stained by the Papanicolaou (Luna, 1968) and Wright Giemsa methods and for nonspecific-esterase reactivity (Williams *et al.*, 1977).

Culture assays for colony forming cells (CFCs)

Cells were cultured as described by Hamburger & Salmon (1977). One ml underlayers, containing enriched McCoy's 5A medium in 0.5% agar were prepared in 35 mm plastic Petri dishes (Falcon 1008). Cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium (GIBCO, Grand Island, N.Y.) with 15% horse serum (Sterile Systems, Logan, Utah). Each culture received 5×10^5 cells in 1 ml of agar-medium mixture. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator.

Scoring of cultures

Cultures were examined with a Zeiss inverted phase microscope at 100 and 160 × magnification. Colony counts of coded plates were made between 10 and 21 days after plating. Aggregates of $\geqslant 30$ light refractile cells were counted as colonies. The 0.3% agar layer, containing the colonies, was fixed and dried onto microscope slides as described by Salmon & Buick (1979). Slides were stained with Papanicolaou stain for morphology and for nonspecific esterase, periodic-acid Schiff and mucicarmine reactivity (Luna, 1968).

Depletion of Adherent cells

Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air in 100 mm plastic tissue culture dishes (Falcon 3003) at a concentration of 2×10^6 cells ml^{-1} in McCoy's 5A medium containing 10% autologous effusion fluid. Nonadherent cells were removed and the adherent cell layer washed twice with 5 ml of 0.002% EDTA-saline. The washings were pooled with the nonadherent fraction. Nonadherent cells contained $5 \pm 3\%$ macrophages based on morphology and NSE stains. The washed adherent layer was then removed with a rubber policeman. Cell yields were 60–85% of input values. Macrophage yields were in the same range. There was no preferential loss of

any cell type after overnight incubation. Viabilities were 85% of that observed before overnight culture. In cases where adherent cells were used as a feeder layer, a known number of adherent cells was suspended in the bottom layer of enriched McCoy's 5A media in 0.5% agar. The nonadherent cell suspensions were then overlaid in 0.3% agar.

Irradiation

Cells were irradiated in suspension with 10 Gy (unless otherwise specified) using a ^{137}Cs irradiation source (5 Gy min^{-1}) (Gammator B, Parsnippany, NJ).

Treatment of adherent cells with monoclonal antibodies

Adherent cells were resuspended at 10^6 cells in 1.0 ml McCoy's 5A medium supplemented with 10% FBS. The cell suspension was incubated with 1.0 ml of antibody at an appropriate dilution at 4°C for 1 h, washed × 3 with HBSS and then incubated at 37°C for 45 min with 1.0 ml of a 1:15 dilution of baby rabbit serum (Pelfrez, Rogers, AR) pretested for the absence of heterospecific antibodies as a source of complement (C). Cells were washed × 3 with HBSS, recounted, and cell kill assessed by vital dye staining. The appropriate number of treated cells was then added to the bottom layer in 35 mm culture dishes as described. The numbers of treated cells added were based on the original cell number present before antibody treatment. This was done to keep the number of antigen-negative cells constant in control and treated groups. Control cells were incubated with complement only.

Monoclonal antibodies

The OK panel of hybridoma monoclonal antibodies (OKT3, OKT4, OKT8) used in these studies were obtained from Ortho Pharmaceutical Corp., (Rartitan, NJ). In brief, the OKT3 antibody identifies a T cell antigen present on the majority of mature circulating T cells. The OKT4 antibody identifies the T-cell subset that provides helper/inducer function in T-T, T-B, and T-macrophage interactions. The OKT8 antibody identifies the T-cell subset that provides cytotoxic suppressor function in these cell–cell interactions. Another pan-T cell antibody, T101 was used in the experiments (Hybritech Corp, La Jolla, CA). In all cases, the cytotoxic activity of each antibody was confirmed using ^{51}Cr release assay (Mishell & Shiigi, 1980) against isolated peripheral blood T and B lymphocytes. Concentrations of antibody used in these experiments caused 85% of maximal release of ^{51}Cr from T cells and had no substantial

activity on B cells. Release in the presence of C alone was ~5%.

Depletion of E-rosetting T lymphocytes

T lymphocytes were isolated by the method of Pellegrino (1976). Cells (5×10^6ml) were mixed with AET (2-aminoethylisothiouronium bromide, Sigma, St. Louis, MO) treated sheep erythrocytes (SRBC). The rosetted cells were then subjected to sedimentation through Ficoll-Hypaque. E negative cells were recovered at the interface and E positive cells in the pellet. After lysis of SRBC with 0.83% Tris-buffered ammonium chloride pH 7.2, E positive cells were used for further study. An aliquot of E positive and E negative cells was then irradiated and allowed to adhere to plastic dishes to isolate adherent cells.

Statistical analysis

The probability of differences between samples being statistically significant was determined by the use of the two-tailed Student's t-test. Four or 5 plates were scored per point. The results are expressed as mean ±s.e.

Results

Effect of irradiation on the ability of adherent cells to support tumour colony growth

We examined the effect of irradiation on the ability of adherent cells to support the growth of tumour colonies. Sufficient numbers of adherent cells were successfully isolated from effusions of 19/35 patients with adenocarcinoma of the ovary, breast or colon, or melanoma. Depletion of adherent cells resulted in a loss of colony forming capacity by the residual nonadherent tumour cells (Table I). In 18/19 cases, addition of 2×10^5 adherent cells to underlayers of cultures containing only nonadherent cells significantly increased the number of colonies observed.

One group of adherent cells from each evaluable sample was exposed to 10 Gy and the ability of irradiated cells to support growth of NA tumour cells compared to controls. The results (Table I) indicate that in 17/19 cases irradiated adherent cells supported the growth of more tumour colonies than untreated adherent cells. In 2 cases, there were no significant differences between the number of colonies observed in the presence of irradiated or untreated adherent cells.

Effect of increasing doses of irradiation

Adherent cells were treated with 2.5–40 Gy to

Table I Effect of irradiation on the ability of adherent cells to support tumour colony growth

Tumour Type		UF	NA	NA+ADH (untreated)	NA+ADH (irradiated)
Ovarian		100±12	36±4	92±16	488±20
		216±12	95±8	516±32	876±40
		N.T.	64±8	172±20	292±16
		236±12	44±2	130±20	158±10*
		552±16	144±12	436±8	598±12
		156±10	40±8	120±8	180±16
		172±6	28±1	156±16	170±8*
Breast		21±6	4±4	48±8	200±12
		133±10	40±4	60±8	180±26
		172±12	32±8	100±4	208±8
		80±6	12±4	60±7	160±4
		120±12	6±6	160±24	220±6
		N.T.	52±8	116±12	172±20
Colon		208±8	12±4	140±10	200±8
,,		160±8	80±5	160±10	320±14
Melanoma		430±20	120±6	464±8	700±28
,,		358±16	140±10	410±20	724±20
Unknown		N.T.	98±8	160±12	652±48
,,		130±4	4±4	28±4	120±4

Column header note: No. of colonies/5×10^5 cells

UF = Unfractionated (cells incubated overnight at 4°C in McCoys 5A media and 10% autologous effusion fluid); NA = nonadherent; NA+ADH untreated = nonadherent cells with adherent untreated (2×10^5) cells in the underlayer; NA+ADH irradiated = nonadherent cells with adherent cells (2×10^5) treated with 10 Gy. Effusions were depleted of adherent cells as specified in the text. Either treated or untreated adherent cells were added back to cultures of nonadherent cells as described.
The mean number of colonies ±s.e. are presented.
NT = not tested.
* = Differences between untreated and irradiated groups were not statistically significant ($P \geq 0.05$).

define the dose-dependence of the enhancing effect. Figure 1 demonstrates that as few as 2.5 Gy significantly increased the ability of adherent cells to support tumour colony growth. A maximal enhancement was reached between 5–10 Gy. Doses >20 Gy slightly but significantly decreased the ability of adherent cells to support tumour colony growth.

Effect of depletion of adherent T-cells

Although adherent cells usually consist of >90% macrophages (Buick et al., 1980; Hamburger & White, 1982), variable numbers of lymphocytes were present in the adherent population (Table II). We and others have demonstrated (Domagala et al., 1978; Haskill et al., 1982) that the majority of
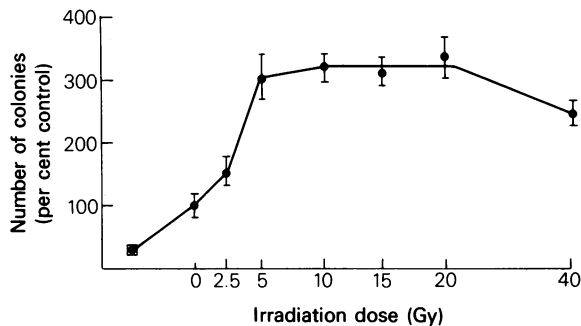
Figure 1 Effect of increasing doses of irradiation on the ability of adherent cells to support tumour colony growth. Adherent cells (2×10^5) from 8 patients were treated with increasing doses of irradiation and then added to cultures containing nonadherent tumour cells. The results from each specimen were normalized to percent control. The number of colonies observed in the presence of unirradiated adherent cells was equal to 100%.

Table II Morphological assessment of adherent cells derived from malignant effusions

Tumour Type	Macrophage/Monocyte	Lymphoid ($OKT3^+$ $OKT3^-$)		Tumour	Meso-thelial	PMN
	80	9	2	5	1	3
	92	7	0	0	1	0
Ovarian	88	8	2	0	2	0
	68	25	5	0	0	2
	80	11	0	9	0	0
Colon	73	8	3	11	0	5

Counts were based on the mean of 2 slides (500 cells/slide).

the lymphocytes in malignant effusions are T-cells. Radiosensitive suppressor T cells have been demonstrated to interact with macrophage-monocytes in a variety of immune responses (Siegel & Siegel, 1977) and in the control of haematopoietic colony growth (Torok-Storb *et al.*, 1981). Therefore, we interpreted our initial results as indicating that the enhanced ability of irradiated adherent cells to support tumour growth was due to inactivation of T cells that may participate in the arrest of tumour colony growth. Adherent cells were depleted of T-cells by the use of a pan-T monoclonal antibody and C. Antibody-treated

adherent cells or untreated adherent cells (2×10^5) were then added to the agar underlayers as described. The results from studies of 9 patients are shown in Table III. In 8/9 cases, the number of tumour colonies observed in the presence of T depleted-unirradiated adherent cells was significantly less than the number observed in the presence of irradiated adherent cells. Therefore, depletion of T cells by antibody-mediated cytolysis was not as effective as irradiation in enhancing tumour colony growth.

Table III Effect of T cell depletion on the ability of adherent cells to support tumour colony growth

Tumour type	No. of colonies/5×10^5 cells			
	NA	NA+ADH untreated	NA+ADH (T101+C)	NA+ADH (irradiated)
	4 ± 4	48 ± 8	100 ± 6	200 ± 12
Ovarian	144 ± 12	436 ± 8	178 ± 10	598 ± 12
	28 ± 4	156 ± 16	136 ± 8	$170 \pm 8*$
	32 ± 8	100 ± 4	146 ± 6	208 ± 8
Breast	12 ± 4	60 ± 7	108 ± 15	160 ± 4
	4 ± 4	48 ± 8	120 ± 12	200 ± 12
Colon	12 ± 4	140 ± 40	108 ± 4	200 ± 8
,,	80 ± 5	160 ± 10	80 ± 7	320 ± 14
Melanoma	120 ± 16	464 ± 8	352 ± 20	700 ± 28

UF = unfractionated. NA = nonadherent; NA+ADH untreated = nonadherent cells with adherent untreated cells (2×10^5) in the underlayer; NA+ADH (T101+C) = nonadherent cells with adherent cells (2×10^5) treated with a pan-T antibody (T101) and C'; NA+ADH irradiated = nonadherent cells with adherent (2×10^5) cells treated with 10 Gy.

Effusions were depleted of adherent cells as described in the text.

Either treated or untreated adherent cells were added back to cultures as described.

The mean number of colonies ±s.e. are presented.

*Differences between untreated and irradiated groups were not statistically significant ($P \geq 0.05$).

We also investigated whether the irradiation-induced enhancement of tumour colony growth was dependent on the presence of T cells. Adherent cells were isolated as described and divided into two groups. One group was treated with the pan-T T101 antibody. Half the cells in each group were subsequently irradiated. Table IV summarizes data from 9 patients (6 ovarian, 3 breast). The results of

Table IV Effect of T cells on the ability of irradiated macrophages to support tumour colony growth

% Control number of colonies/5×10^5 cells		
	T cells present	T cells removed
Untreated adherent cells	322 ± 42	477 ± 76
Irradiated adherent cells	658 ± 39	205 ± 24

Results are expressed as % of control colony growth (non-adherent cells only) and represent mean ±s.e. of 9 experiments. Adherent cells were isolated as described. Groups were either irradiated and/or depleted of T cells by treatment with a monoclonal antibody, as indicated in the text. Adherent cells were then added to underlayer and 5×10^5 autologous non-adherent tumour cells added to the agar overlayer.

Figure 2 The effects of adherent cells depleted of T lymphocytes or T lymphocyte subsets on the growth of tumour colonies from nonadherent tumour cells. Bars represent mean ±s.e. In the one of 4 similar experiments, adherent cells were isolated from effusions and pretreated with C and one of 3 monoclonal antibodies (OKT3, T4, T8). Adherent cells (2×10^5) were then added to cultures of 5×10^5 nonadherent cells as described. Adherent cells treated with OKT3 and C are designated ADH−T3 etc.

each experiment were normalized to percent of control observed in the presence of non-adherent cells. The average increase in growth over that observed with nonadherent cells only was calculated for each group. Depletion of T cells by antibody from control adherent cells resulted in variable increases in tumour growth. In contrast, the increased stimulating activity of the irradiated macrophages was found only when T cells were present. This indicates that the increased tumour colony enhancing activity of the irradiated macrophages was T cell dependent.

Effect of depletion of T cell subsets

We interpreted the above findings as indicating that the effect of eliminating radiosensitive T cell populations that may suppress tumour CFC growth was obscured by removing the entire population of T lymphocytes. Another subpopulation of T cells that enhance tumour colony growth may have been eliminated. We therefore tested the ability of T cell subsets and macrophages to potentiate growth of tumour CFCs.

Adherent cells were depleted of either all T cells (OKT3+), helper T cells (OKT4+), or suppressor cells (OKT8+), and the ability of the residual adherent cells to support growth of tumour colonies examined. The results from 4 patients are shown in Figure 2. Again, depletion of adherent cells decreased colony formation. Addition of adherent cells restored the colony growth. Irradiation greatly increased the ability of adherent cells to enhance colony growth. Depletion of OKT4+ cells from unirradiated adherent cells resulted in a small but not statistically significant decrease in colony formation as compared to untreated adherent controls. Depletion of OKT8 positive cells,
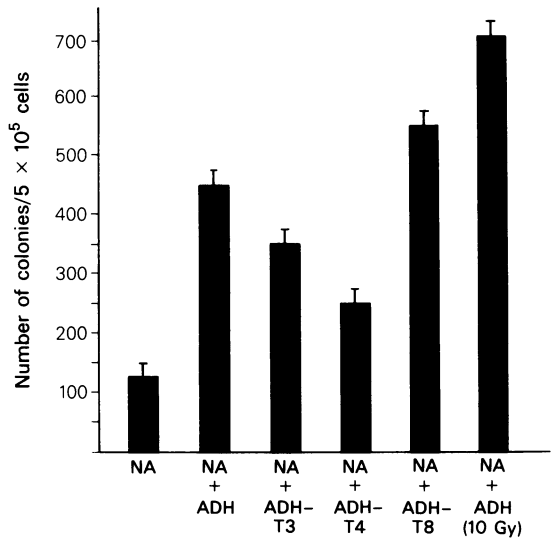
however, resulted in greatly increased colony formation.

Selective effect of irradiation on the ability of adherent cells to enhance colony formation

We wished to demonstrate directly that the irradiation-induced augmentation of the ability of adherent cells to support tumour colony formation was due to a selective effect on T cells. We therefore isolated and selectively treated macrophages and lymphocytes. Adherent cells were isolated as described and depleted of T cells by E-rosetting prior to irradiation. The cell populations were then reconstituted and assayed for their ability to support tumour colony formation. The results of one representative experiment (out of 4), using cells from a patient with ovarian cancer, are shown in Table V. Irradiation of macrophages alone did not increase the ability of adherent cells (macrophages and lymphocytes) to support tumour colony formation. By contrast, irradiation of lymphocytes alone increased the number of colonies to values observed when the reconstituted (irradiated macrophages and irradiated lymphocytes) were used. While T-cell depleted adherent cells could

Table V Effect of irradiation of isolated macrophages and lymphocytes on tumour colony formation

Cell combination	Cell irradiated	No. of colonies $(5 \times 10^5$ cells)
M + L	None	96 ± 8
M + L	Both	168 ± 8
M (10 Gy) + L	Macrophage	100 ± 4
M + L (10 Gy)	Lymphocyte	188 ± 8
M alone	None	120 ± 16
M alone	Macrophages	68 ± 8

Adherent cells were separated into macrophages (M) and T lymphocytes (L) as described. The isolated populations were irradiated, recombined and added to cultures of NA tumour cells.

support growth, the enhanced ability of the irradiated macrophages to support growth was T cell dependent.

Discussion

The results of this study confirm our previous findings indicating that adherent and phagocytic cells are required for optimal *in vitro* tumour colony growth (Hamburger *et al.,* 1978). We now report that in 17/19 cases, irradiation enhanced the ability of adherent cells to support tumour colony formation. The irradiation induced enhancement may have been due to several factors. However, one of the more plausible explanations is that cells that suppress colony growth, either directly or indirectly, may have been inactivated.

Inactivation of radiosensitive (5–10 Gy) lymphoid suppressor cells has been demonstrated in a number of immunological systems (Siegel & Siegel, 1977; Saiki & Ralph, 1982). In most cases, the lymphoid cell is a T lymphocyte. This finding, plus the fact that the majority of adherent lymphoid cells in effusions are T lymphocytes, (Haskill *et al.,* 1982) led us to hypothesize that a major effect of irradiation was to inactivate a suppressor T lymphocyte. There is evidence that T cells may either directly suppress tumour growth (Gupta *et al.,* 1978; Allavena *et al.,* 1982), or modulate secretion of cytostatic products of macrophages (Cameron & Churchill, 1979; Kleinerman, 1981). Similarly, T cells modulate the ability of macrophages to produce acidic isoferritins that directly inhibit granulocyte-macrophage colony growth (Broxmeyer, 1981).

Attempts were made to determine whether the potentiation of tumour colony growth by irradiation was mediated by T lymphocytes. This was most simply tested by rigorously eliminating T

lymphocytes from the adherent cell population by antibody-mediated cytolysis and then determining whether antibody treatment or irradiation produced equivalent effects. The results indicate that depletion of T cells did not increase the colony stimulating ability of the adherent cells as much as did irradiation. However, we felt it was still likely that their irradiation effect was mediated by T lymphocytes. This was based on the fact that the ability of macrophages to secrete CSFs for haematopoietic colony forming cells is unaffected by irradiation (Williams *et al.,* 1981). We therefore postulated that treatment with the pan-T antibody eliminated both "suppressor" and "helper" subsets of T cells resulting in no overall change in tumour colony growth.

The existence of T lymphocytes that may augment secretion of CSFs for tumour cells was further supported by the finding that the increased stimulating activity of irradiated macrophages was found only when irradiated T cells were present. This indicates that the increased tumour colony enhancing activity of the macrophages was T cell dependent. This is reminiscent of the modulation of erythroid (Torok-Storb *et al.,* 1982) and granulocyte (Bagby *et al.,* 1982) colony formation in soft agar and tumour growth *in vivo* (Gabizon *et al.,* 1980) that is dependent on T cell-macrophage collaboration.

We postulated that different subsets of T cells may be enhancing or limiting the growth of tumour colonies in collaboration with macrophages. The existence of easily separable subsets of T lymphocytes capable of either enhancing or inhibiting growth of transplantable animal tumours has been demonstrated (Small & Trainin, 1976; Blazar *et al.,* 1978). This hypothesis was directly tested by first treating the T cell population with either T4, T8 or T3 antibodies and C. These antibodies eliminate helper, suppressor, or all T cells respectively. The results of this study indicate that elimination of T4 helper subsets did not significantly decrease colony formation below that observed with untreated adherent cells. Growth was still significantly greater than that observed from nonadherent cells only. Treatment with T8 antibody resulted in increased levels of colonies above untreated controls, although the number of colonies was still somewhat lower than those observed with irradiation.

Further experiments in which either isolated T cells or macrophages were irradiated supported the hypothesis that the irradiation induced effect was via a T-lymphocyte. Irradiation of isolated macrophages alone did not enhance the ability of adherent cells (irradiated macrophages and untreated T lymphocytes in combination) to

support tumour colony growth. In contrast, when irradiated T cells were added to untreated macrophages, increased colony formation over that observed in the presence of the reconstituted untreated macrophage-lymphocyte combination was observed.

Isolated T-depleted irradiated macrophages could support colony growth but the irradiation induced enhancement of colony growth was observed only when T cells were present. One interpretation of these findings is that a radiosensitive T cell may both inhibit tumour colony formation (directly or via macrophages) and also inhibit the production of growth enhancing factors by radioresistant T cells.

The results of these studies demonstrate that both adherent lymphocytes and macrophages can affect the growth of human tumour colonies in soft agar. Macrophages have been demonstrated to either enhance (Hamburger *et al.*, 1978) or inhibit (Haskill *et al.*, 1975) colony formation. Interacting subsets of T cells may modulate the ability of macrophages to produce colony stimulating or colony inhibiting factors. It is likely that individual patients can be characterized by a unique balance of tumour cell-accessory cell interactions at any time in the natural history of their disease. As the characterization of growth inhibiting or enhancing populations is limited by difficulties in obtaining and purifying adequate numbers of cells, future work should be directed towards obtaining and expanding these accessory cell populations.

The demonstration that manipulation of tumour-accessory cell interactions *in vitro* influences tumour cell growth may have implications for clinical treatment. The studies presented here are consistent with the hypothesis that many human tumours are to varying extents dependent on immune cells (Prehn, 1977). Different subsets of macrophages and lymphocytes may have opposing effects on tumour cell survival and proliferation. In addition, the same populations that are stimulatory at low concentrations may be inhibitory at higher concentrations. Thus, alterations in either subset type, number, or activation state of immune cells by biological response modifiers or monoclonal antibodies may prove beneficial. Further proof that many human tumours are influenced by macrophage and lymphocytes may facilitate development of effective immunological approaches to therapy.

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