Depressed Natural Killer Cell Activity in Patients with Hepatocellular Carcinoma

In Vitro *Effects of Interferon and Levamisole*

K. SON, BSc; M. KEW, MD, PhD, FRCP, AND A. R. RABSON, MB BCh, MRC (Path)

Enriched lymphocytes from patients with primary hepatocellular carcinoma showed considerably reduced natural cytotoxicity against two established hepatoma cell lines and against the myelogenous derived cell line K562, as compared to lymphocytes from normal volunteers or from patients with various nonmalignant liver diseases. The serum of hepatoma patients did not effect normal or patient NK cell activity. When lymphocytes from patients or controls were treated for three hours with human leukocyte interferon, NK cell activity was significantly improved. Levamisole did not produce a significant increase in normal NK cytotoxicity but did improve the killing of lymphocytes from patients with hepatocellular carcinoma.

Cancer 50:2820–2825, 1982.

Natural killer (NK) cells are normal unprimed mononuclear cells which have cytotoxic activity against a variety of tumor target cells. They are found in all normal donors and are believed to play an important role in the defence against tumor cells functioning either as a form of surveillance against newly arising malignant clones, or as a mechanism by which the accessible cells of established tumors are destroyed.[1,2] Studies on NK activity of lymphocytes isolated from animals with experimental tumors or humans with spontaneous tumors have yielded variable results. Pross and Baines[3] reported depressed NK activity in patients with ovarian cancer, especially those with advanced disease, and Gerson,[4] who studied patients with a variety of tumors found depressed NK cell activity but mainly in patients with neuroblastoma. Depressed NK activity appeared to be correlated with extent of disease but not with the size of the primary tumor, since some patients with large localized tumors had high levels of NK activity. Patients with early, operable breast cancer have normal NK activity[5,6] and studies on patients with lung cancer have yielded conflicting results.[7,8]

From the South African Medical Research Council Human Cellular Immunology Unit, School of Pathology of the University of the Witwatersrand and the South African Institute for Medical Research. Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa.

Supported in part by the Medical Research Council and National Cancer Association of South Africa.

Address for reprints: A. R. Rabson–SAIMR, Department of Immunology, P. O. Box 1038, Johannesburg, 2000 South Africa.

Accepted for publication October 19, 1981.

This study investigated NK cell activity in a group of patients with hepatocellular carcinoma (HCC), an extremely common malignant tumor in black South African men. Two different established hepatoma cell lines as well as the myelogenous leukemia cell line K562 were used as targets for NK activity. The depressed NK activity which was observed could be partly corrected by interferon and, to a lesser extent, by levamisole.

Materials and Methods

Patients

Thirty-six patients with histologically proved HCC were studied, and in three of these patients tests were performed twice. All patients were blacks, and none were receiving any form of therapy at the time of study. There were 34 men and two women; their ages ranged from 19–56 years (mean, 42 years). The clinical condition (performance status), extent of tumor burden, as judged by the size of the defect or defects on hepatic scintiscanning, serum alpha-fetoprotein (AFP) and hepatitis-B virus surface antigen (HBsAg) status, and biochemical tests of liver function (serum bilirubin, albumin, alkaline phosphatase, and aminotransferase concentrations and prothrombin index) were determined in each patient for correlation with NK cell activity. Ten patients with various forms of acute and chronic liver disease were also studied. This group comprised nine men and one woman with a mean age of 47 years and included patients with acute viral hepatitis, cryptogenic

0008-543X/82/1215/2820 $1.10

cirrhosis, alcoholic cirrhosis, chronic active hepatitis, and amebic liver abscess. Normal healthy laboratory workers served as controls.

Preparation of Human Peripheral Lymphocytes

Peripheral lymphocytes from patients or controls were separated from heparinized blood by centrifugation on a Ficoll-Hypaque density gradient. This method yielded more than 95% mononuclear cells, and viability was greater than 90% as determined by trypan blue dye exclusion. The cells were washed twice in Hank's balanced salt solution (HBSS) and resuspended in RPMI 1640 with 10% heat inactivated fetal calf serum (RPMI-FCS). Mononuclear cells were depleted of adherent cells by incubation on plastic tissue culture petri dishes at a cell concentration of 1.5×10^6 cells/ml for 1 hour at 37°C in a humidified 5% CO_2 atmosphere. The nonadherent lymphocytes were suspended in RPMI-1640 with 10% heat inactivated human AB serum and used in the chromium release assay (CRA).

Cell Lines

Two established cell lines derived from human HCC and a myeloid cell line were used as target cells in the CRA assay. PLC/PRF/5 is a cell line established by Alexander *et al.*[9] from a man with HCC and hepatitis B surface antigenemia. This line produces a number of liver proteins such as transferrin, α-1-antitrypsin, ceruloplasmin, C3, C1 inactivator and α-2-macroglobulin, and secretes and expresses HBsAg on the cell membranes.[10] The Mahlavu cell line is an HBsAg-negative human continuous cell line also derived from a patient with HCC but it does not secrete liver proteins.[11] The K562 is derived from a chronic myeloid leukemia cell line[12] highly sensitive to natural cytotoxicity.

Cultures of both HCC cell lines were maintained continuously in 25 ml tissue culture flasks (Lux Scientific Corp.) in RPMI-1640 supplemented with 10% foetal calf serum and added antibiotics, and were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C and subcultured at confluence. Cultures were treated with 0.25% trypsin in Versene buffer for five minutes at 37°C and single-cell suspensions were washed and resuspended in RPMI-FCS at a concentration of 5.0×10^6 viable cells/0.2 ml medium. K562 cells were grown as stationary cultures and subcultured weekly in RPMI-1640 with 10% FCS.

Chromium Labeling of Target Cells

Tumor cells were labelled by incubating with 100 μCi $Na_2\ ^{51}Cr^{0}4$ (Radiochemical Centre, Amersham) for two hours at 37°C in a humidified 5% CO_2 incubator. The labelled target cells were washed five times with HBSS, and resuspended in RPMI with human AB serum. Viable cells as determined by trypan blue dye exclusion were adjusted to 5.0×10^4 cells/ml.

Chromium Release Assay (CRA)

A total of 0.2 ml of labeled target cells were dispensed onto flat bottomed 96 well microtitre plates (Falcon) and allowed to adhere overnight at 37°C in a humidified 5% CO_2 atmosphere. The target cells were washed with RPMI plus AB serum and 0.2 ml effector cells were added in triplicate to the wells. Ratios of effector cells to target cells of 40:1 and 80:1, respectively, were used routinely as preliminary studies indicated maximal cytotoxicity when these two ratios were employed. With every test system, some wells were inoculated with labelled target cells and medium alone to allow calculation of spontaneous ^{51}Cr release. Employing the two HCC cell lines, spontaneous release did not exceed 25–30% of the maximal release obtained over the 18 hour period. For the K562 cell line, spontaneous release did not exceed 10–15%. In addition, in other wells cells were lysed with distilled water to determine maximal release. The microtitre plates were centrifuged at 1000 rpm for three minutes and incubated at 37°C in a humidified 5% CO_2 incubator for 18 hours. Previous experiments indicated that maximal ^{51}Cr release was observed after this time interval. One hundred fifty μl of supernatant was then removed and counted on a Hewlett Packard auto gamma counter.

The percentage ^{51}Cr release was determined for each well and using the mean value of triplicate wells the percentage cytotoxicity calculated according to the following formula:

$$\%\ \text{cytotoxicity} = \frac{\text{sample–spontaneous}}{\text{maximal–spontaneous}}$$

Results were analyzed statistically using the Mann Whitney's U test.

Pretreatment of Lymphocytes with Interferon or Levamisole

Crude IF prepared from human leukocytes incubated with blue tongue virus and standardized to 10,000 IU/ml against an international standard (NIH) was a gift from Professor B. Schoub (National Institute of Virology). Levamisole was obtained from Ethnor Laboratories, Johannesburg. Lymphocytes at a concentration of 2.0×10^6/ml were incubated with or without varying concentrations of interferon or levamisole for a period of three hours. At the end of this incubation period cells were washed three times in HBSS and assayed for cytotoxicity. To exclude the possibility that either of these

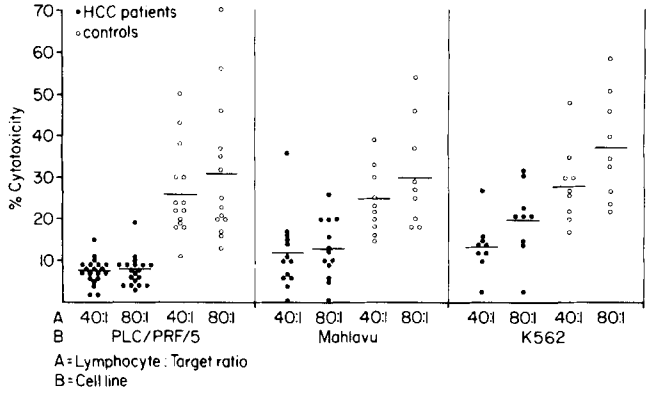

FIG. 1. The percent cytotoxicity produced by enriched lymphocytes from normal controls and HCC patients.

agents had a direct cytotoxic effect, in some experiments they were added directly to target cells and ^{51}Cr release assessed.

Results

Lymphocytes from patients and controls were added to target cells at two different ratios (40:1 and 80:1). As is indicated in Figure 1, at both these ratios considerably reduced cytoxicity was produced by HCC patients' lymphocytes as compared to normals. Cytotoxicity by normal lymphocytes was greater when an 80:1 ratio was employed, but with lymphocytes from HCC patients similar depressed cytotoxicity was observed with both cell ratios. Depressed cytotoxicity was observed when the PLC/PRF/5, the Mahlavu or the K562 cell lines were employed and similar results were obtained when either unseparated mononuclear cells or adherent cell depleted mononuclear cells were employed (results not shown). NK activity was reassessed in three patients at intervals of three weeks and the activity remained unchanged (details not shown). No correlation could be demonstrated between age, performance status, size of the tumor mass or masses, or tests of liver dysfunction, and NK cell activity in individual patients. AFP was present in high concentration in the serum of 23 of the patients (64%) and HBsAg was detected in 19 patients (53%). There was no difference in the percentage cytotoxicity of lymphocytes between AFP-positive and AFP-negative patients or between HBsAg-positive and HBsAg-negative patients.

To assess whether the serum of patients with HCC had any effect on NK activity by normal or patients' cells, serum from these patients was added to both normal and patients' lymphocytes. As is indicated in Figure 2, the depressed NK activity of HCC patients' lymphocytes was not altered by treatment with patients' serum. Furthermore, this serum had no effect on normal NK cell activity.

NK cell activity was also assessed in a group of patients with varying degrees of liver damage caused by disease other than HCC. As is shown in Figure 3, there was no significant difference in this group as compared to controls.

To assess the effect of human IF on NK cell activity, HCC lymphocytes were incubated with varying concentrations of IF for three hours prior to testing for cytotoxicity employing the PLC/PRF/5 cell line as target. Interferon at all doses employed increased the percentage cytotoxicity of HCC lymphocytes with a maximal

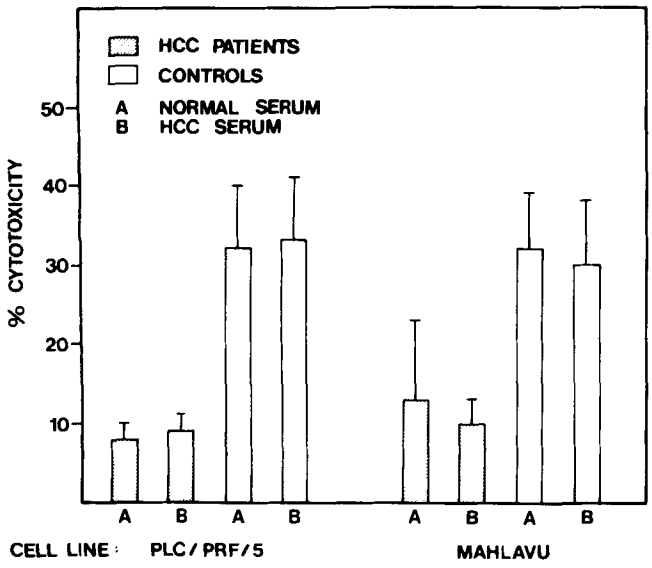

FIG. 2. The percent cytotoxicity produced by enriched lymphocytes from normal and HCC patients in the presence of normal or HCC serum.

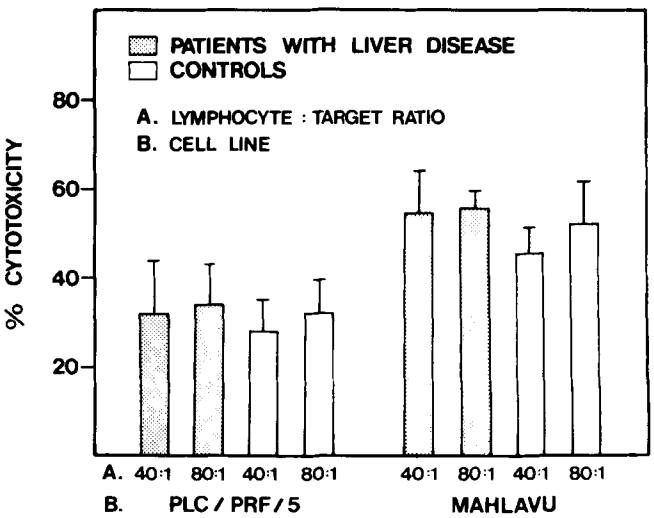

FIG. 3. Percent cytotoxicity of lymphocytes from normals and patients with other forms of liver disease.

effect being observed when a dose of 500 units/ml of interferon was employed (Fig. 4). A maximal effect of the IF was observed when cells were pretreated with it for a period of three hours prior to cytotoxicity testing (Fig. 5) and the effect was observed when both normal and HCC lymphocytes were employed. When normal MN cells were pretreated with levamisole for three hours prior to cytotoxicity testing, some increase in NK cell activity was observed with a maximum effect being produced when a dose of 60 μg/ml was employed (Fig. 6). The results, however, were not statistically significant. When lymphocytes from patients with HCC were pretreated with 60 μg/ml levamisole a significant increase in NK cell activity was observed with ratios of both 40:1 and 80:1 (Table 1). Neither IF nor levamisole had a direct cytotoxic effect on tumor target cells (results not shown).

Discussion

Some controversy exists as to the state of NK cell activity in patients with cancer. This study clearly indicates considerably reduced NK cell cytotoxicity from peripheral blood mononuclear cells of untreated patients with HCC employing two HCC cell lines as targets. Both of these cell lines have been used by others in cytotoxicity testing. The effect was not due to a serum factor since normal NK cell activity could not be abrogated by incubation with serum from the HCC patients. Because diminished killing was also observed against a myeloid cell line (K562) and an esophageal carcinoma cell line (results not shown) the reduced NK cell activity was not specific. Patients with a variety of other acute and chronic liver diseases, including some with large space-occupying lesions (amoebic liver abscesses) showed normal NK cell activity, indicating that involvement of the liver *per se* was not the cause of the depressed NK cell function.

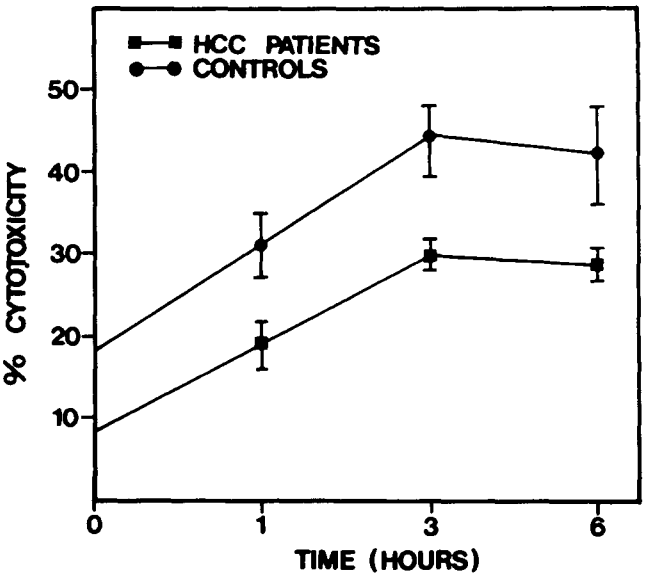

FIG. 5. Effect of 500 Units/ml of interferon on the NK cell activity of normals and HCC patients (mean ± SEM of 5 experiments, E:T ratio 40:1).

It had been suggested that the degree of depression of cytotoxicity in cancer patients is related to tumor burden, and that natural killing is high when the tumor load is large and decreases when the tumor burden is lessened.[8] In the group of patients examined in this study, tumor load was not assessed formally, but as all patients had massive enlargement of the liver with one or more large defects visualised on liver scan, it is likely that tumor mass was extremely large. Due to the rapid growth rate of these tumors, all patients had advanced disease at the time of study, and it was not possible to test NK cell activity in any patients with early disease. As every patient examined had considerably reduced natural cytotoxicity, no correlation could be detected between a variety of clinical parameters of disease and

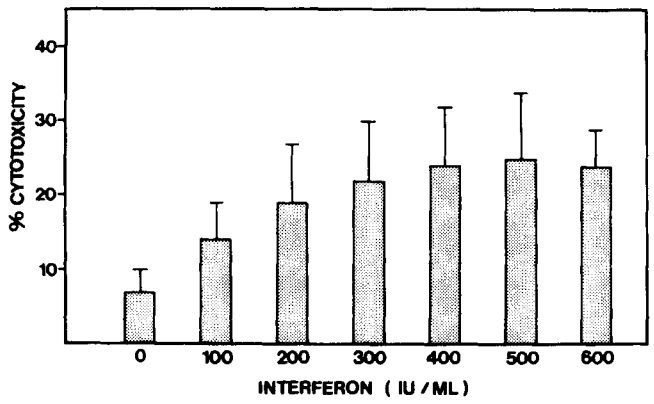

FIG. 4. The effect of increasing concentrations of interferon on the NK cell activity of HCC patients (mean of 10 experiments, E:T ratio 40:1).

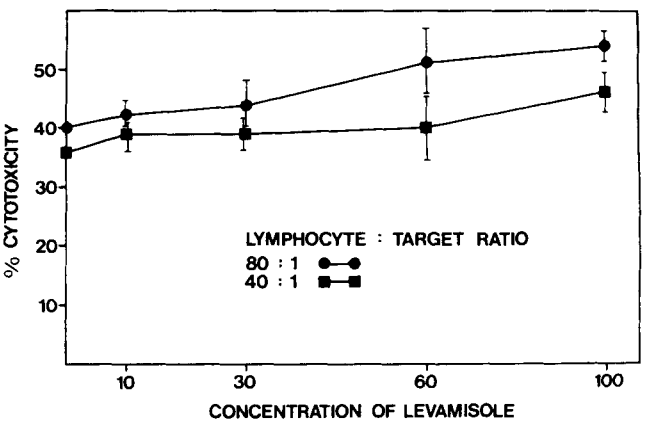

FIG. 6. Effect of increasing doses of levamisole on normal NK Cell activity (mean ± SEM of 5 experiments).

TABLE 1. The Effect of Levamisole (60 μg/ml) on NK Cell Activity of Normals and Patients with HCC (Mean and SEM of 10 Experiments)

	Hepatoma patients				Controls			
Levamisole	Without		With		Without		With	
E:T ratios	40:1	80:1	40:1	80:1	40:1	80:1	40:1	80:1
Percentage cytotoxicity	3.0	3.0	7.0	18.0	30.0	33.0	43.0	42.0
	14.0	21.0	24.0	33.0	20.0	22.0	27.0	29.0
	12.0	21.0	28.0	44.0	22.0	24.0	27.0	42.0
	16.0	31.0	16.0	35.0	26.0	51.0	36.0	71.0
	12.0	23.0	22.0	28.0	27.0	35.0	21.0	39.0
	10.0	15.0	16.0	24.0	23.0	27.0	27.0	35.0
	10.0	8.0	11.0	10.0	13.0	20.0	12.0	20.0
	15.0	10.0	16.0	18.0	22.0	24.0	21.0	22.0
	9.0	18.0	16.0	21.0				
	4.0	5.0	6.0	10.0				
Mean	11.0	16.0	16.0	24.0	23.0	27.0	27.0	35.0
±SEM	1.0	3.0	2.0	3.0	2.0	6.0	4.0	8.0
P value			<0.005	<0.005			NS	NS

NK cell activity. The patients in this study were receiving no therapy at the time of examination, and although the cause of the NK cell depression in these patients is not known, a number of possibilities exist. NK cells in the peripheral blood could become depleted due to selective recruitment or entrapment at the tumor site in which case NK cell activity of tumor infiltrating lymphocytes should be high. However, in a number of studies the lymphocytes infiltrating human tumors or found in draining lymph nodes have been shown to have low NK activity.[4,6,13] It is also possible that the activity of NK cells could be depressed by factors released from the tumor or by suppressor cells of NK cells such as those observed in the spleens of C. Parvum treated mice.[14] Alternatively, it is possible that NK cell activity requires constant priming by interferon, a process which could be disturbed in malignant disease.

In the current study human leukocyte interferon caused considerable enhancement of NK cell activity at all doses employed, and this observation has previously been noted by a number of other workers employing both *in vitro* and *in vivo* models.[15-17] Although the reasons for this augmentation are not clear, a number of possibilities have been suggested, the conventional view being that interferon 'activates' NK cells in much the same way as lymphokines activate macrophages. Ortaldo *et al.*[18] suggest that interferon binds to receptors on NK or pre-NK cells and thereby triggers new messenger RNA production and then new proteins involved in the cytotoxic activity of the NK cells. Alternatively, interferon could activate NK cell precursors leading to the maturation of these cells, and Saksela *et al.*[19] have shown that human MN cells depleted of functional NK activity by selective adsorption to target cells, could be induced to become cytolytic after treatment with interferon. Another possibility is that interferon might mediate its effect by acting on various regulatory cells of NK activity, although interferon has been shown to be effective even in the functional absence of T- and B-cells or macrophages.[20] There is however, some evidence to indicate that NK cell activity is under the control of regulatory lymphocytes[21,22] and it is possible that both interferon and levamisole may mediate their effects on NK cell activity via these cells. Ito *et al.*[23] have demonstrated the presence of natural interferon-producing-cells which are Ia-negative macrophages. The finding that levamisole partially corrects the abnormal NK cell activity could indicate that it is acting on this cell, as levamisole has been shown by others to affect primarily mononuclear phagocyte function.[24] This antihelminthic drug has been shown to potentiate both *in vitro* and *in vivo* immune responses in animals and man and to restore cellular function when this is depressed.[25] In this study significant potentiation of NK cell activity could only be observed when HCC patient cells were employed, as levamisole had only a modest and not statistically significant effect on normal cells. Although a beneficial role in cancer has not been definitely established for levamisole, it does appear to control the metastatic spread of tumor cells to the lungs and other organs.[26,27] Although it is tempting to suggest that this beneficial effect could be mediated via the effect of levamisole on NK cells, it should be stressed that the stimulation of NK cell activity observed in this study was modest and required greater concentrations of levamisole than that possible employing therapeutic doses. Furthermore, the drug has been shown to augment several other processes involved in the inflammatory or the immune response[17]

and the potentiation of NK cell activity observed in this study could represent one more facet of a drug acting at numerous levels of the immune response.

REFERENCES

1. Takasugi M, Mickey MR, Terasaki PI. Reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer Res* 1973; 33:2898–2902.
2. Pross HF, Baines MG. Spontaneous human lymphocyte mediated cytotoxicity against tumor target cells: IV. A brief review. *Cancer Immunol Immunother* 1977; 3:75–85.
3. Pross HF, Baines MG. Natural killer cells in tumor-bearing patients. In: Herberman RB, ed. Natural cell-mediated immunity against tumors. New York: Academic Press, 1980; 1063–1072.
4. Gerson JM. Systemic and in situ natural killer activity in tumor-bearing mice and patients with cancer. In: Herberman RB, ed. Natural cell-mediated immunity against tumors. New York: Academic Press, 1980; 1047–1062.
5. Cannon GB, Bonnard GD, Djeu J, West WH, Herberman RB. Relationship of human natural lymphocyte-mediated cytotoxicity of breast-cancer derived target cells. *Int J Cancer* 1977; 19:487–497.
6. Eremin O. NK cell activity in the blood, tumor-draining lymph nodes and primary tumors of women with mammary carcinoma. In: Herberman RB, ed. Natural cell mediated immunity against tumors. New York: Academic Press, 1980; 1011–1029.
7. Vose BM, Vanky F, Fopp M, Klein E. Restricted autologous lymphocytotoxicity in lung neoplasia. *Br J Cancer* 1978; 38:375–381.
8. Forbes JT, Greco FA, Oldham RK. Natural cell-mediated cytotoxicity in human tumor patients. In: Herberman RB, ed. Natural cell-mediated immunity against tumors. 1980; 1031–1045.
9. Alexander JJ, Bey EM, Geddes EW, Lecatsas G. Establishment of a continuously growing cell line from primary carcinoma of the liver. *S Afr Med J* 1976; 50:2124–2128.
10. Macnab GM, Alexander JJ, Lecatsas G, Bey EM, Urbanowicz JM. Hepatitis B surface antigen produced by a human hepatoma cell line. *Br J Cancer* 1976; 34:509–515.
11. Dienstag JL, Bhan AK. Enhanced *in vitro* cell mediated cytotoxicity in chronic hepatitis B virus infection: Absence of specificity for virus-expressed antigen on target cell membranes. *J Immunol* 1980; 125:2269–2276.
12. Lozzio CB, Lozzio BB. Cytotoxicity of a factor isolated from human spleen. *J Nat Cancer Inst* 1973; 50:535–538.
13. Mantovani A, Allavena P, Sessa C, Bolis G, Mangioni C. Natural killer activity of lymphoid cells isolated from human ascitic ovarian tumors. Int J Cancer 1980; 25:573–582.
14. Savary CA, Lotzova E. Suppression of natural killer cell cytotoxicity by splenocytes from Corynebacterium parvum-injected, bone marrow-tolerant and infant mice. *J Immunol* 1978; 120:239–243.
15. Einhorn S. Interferon and spontaneous cytotoxicity in man. IV. Evidence for interferon as the factor in human interferon preparations causing enhancement of spontaneous cytotoxicity. *J Clin Lab Immunol* 1980; 3:35–38.
16. Einhorn S, Blomgren H, Strander H. Interferon and spontaneous cytotoxicity in man: II. Studies in patients receiving exogenous leukocyte interferon. *Acta Medica Scand* 1978; 204:477–483.
17. Herberman RB, Ortaldo JR, Bonnard GD. Augmentation by interferon of human natural and antibody-dependent cell-mediated cytotoxicity. Nature 1979; 277:221–223.
18. Ortaldo JR, Herberman RB, Djeu JY. Characteristics of augmentation by interferon of cell mediated cytotoxicity. In: Herberman RB, ed. Natural cell-mediated immunity against tumors. New York: Academic Press, 1980; 593–608.
19. Saksela E, Timonen T, Cantell K. Human natural killer cell activity is augmented by interferon via recruitment of "pre-NK" cells. *Scand J Immunol* 1979; 10:257–266.
20. Senik A, Kolb JP, Örn A, Gidlund M. Study of the mechanism for *in vitro* activation of mouse NK cells by interferon. *Scand J Immunol* 1980; 12:51–60.
21. Cudkowicz G, Hochman PS. Regulation of natural killer activity by macrophage-like and other types of suppressor cells. In: Siskind GW, Litwin SD, Weksler ME, ed. Developmental Immunology, Fifth Irwin Strasburger Memorial Seminar on Immunology. New York: Grune & Stratton, 1979.
22. Pollack SB, Emmons SI. Inhibition of human killer cells by autologous lymphocytes. *J Immunol* 1979; 122:718–722.
23. Ito Y, Aoki H, Kimura Y, Takano M, Shimokata K, Maeno K. Natural interferon-producing cells in mice. *Inf Immunity* 1981; 31:519–523.
24. Gordon MY, King JA, Gordon-Smith EC. Abnormal adherent cell function in aplastic anaemia demonstrated using levamisole in cultures for T-lymphocyte colony-forming cells (TL-CFC) *Clin Exp Immunol* 1981; 44:117–123.
25. Symoens J, Rosenthal M. Levamisole in the modulation of the immune response: The current experimental and clinical state. *J Retic Soc* 1977; 21:175–221.
26. Sadowski JM, Rapp F. Inhibition by levamisole of metastases by cells transformed by herpes simplex virus type I. 1975. *Proc Soc Exp Biol Med* 1975; 149:219–222.
27. Fidler IJ, Spitler LE. Effects of levamisole on *in vivo* and *in vitro* murine host response to syngeneic transplantable tumor, 1975. *J Natl Cancer Int* 1975; 55:1107–1112.