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IMMUNOSUPPRESSIVE EFFECTS OF METHYLGLYOXAL-BIS(GUANYLHYDRAZONE) ON MOUSE BONE MARROW AND SPLEEN CELLS AND THEIR ANTAGONISM BY SPERMIDINE*

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Abstract—Changes in immunological function and in the activity of *S*-adenosylmethionine decarboxylase were studied in bone marrow and spleen cells from mice immunized with sheep red blood cells and given multiple doses of methylglyoxal-bis(guanylhydrazone) (CH_3-G) or CH_3-G in combination with spermidine. Inhibition by CH_3-G was found in the complement-dependent and complement-independent immune responses of cells from both tissue sources; however, this inhibition was greater and more persistent in bone marrow than in spleen cells. Co-administration of spermidine with CH_3-G completely prevented this inhibition in the spleen at all the time points studied, whereas in the bone marrow prevention appeared later and was lower in magnitude. The increase in the activity of *S*-adenosylmethionine decarboxylase, a key enzyme in spermidine biosynthesis, was greater in bone marrow than in spleen after the administration of CH_3-G. Co-administration of spermidine with CH_3-G consistently reduced this increase in enzyme activity in spleen but not in bone marrow. In addition to prevention, appropriate scheduling of spermidine subsequent to CH_3-G resulted in reversal of both the immunosuppression and the increase in *S*-adenosylmethionine decarboxylase activity produced by CH_3-G in spleen cells.

Methylglyoxal-bis(guanylhydrazone) (CH_3-G) is an antiproliferative agent which has been used in the treatment of acute myelocytic leukemia [1, 2] and is currently being used for the maintenance in acute myelocytic leukemia (E. Henderson, personal communication). Although the mechanism of cytotoxicity by CH_3-G is still unknown, the prevention of its proliferative toxicity by spermidine [3] suggests that this effect may be related to an interference with polyamine metabolism. CH_3-G has been found to inhibit *S*-adenosylmethionine decarboxylase (SAMDC), a key enzyme involved in the biosynthesis of spermidine [4]. However, administration of this drug *in vivo* has led to an increase in SAMDC activity in various rodent tissues [5]. This was apparently due to an intracellular stabilization of the enzyme as a result of drug binding which was manifested as an apparent increase in enzyme half-life and an eventual increase in enzyme activity. Furthermore, CH_3-G has been shown to compete with spermidine for uptake into cells [6]. Since increases in polyamine levels have been correlated with increases in cellular proliferation [7], the relationships of CH_3-G to polyamines may be related to the antiproliferative action of the drug.

The effectiveness of CH_3-G as a cancer chemotherapeutic agent has been limited by its toxicity to host tissues such as gastro-intestinal epithelium and various cell types within the immune system [2]. Previous studies from this laboratory have shown selectivity in the effects of CH_3-G on immune effector cells within mouse spleen [8], namely the immune response of antibody-forming B cells to sheep red blood cells (SRBC) was inhibited while the responses of phagocytes and non-specific killer cells were not affected as a result of CH_3-G administration. Correspondingly, the uptake of CH_3-G as well as the CH_3-G-related enhancement in SAMDC activity was greater in "B cell"-enriched populations than it was in "T cell"-enriched populations.

This investigation was undertaken to compare the effects of CH_3-G on mouse bone marrow and spleen cells and also to evaluate the antagonistic influence of spermidine on the changes caused by CH_3-G in these tissues. It was found that after the administration of CH_3-G to mice, the increase in SAMDC activity and the inhibition of the immune response to SRBC appeared to be greater and longer lasting in bone marrow than in spleen cells. Co-administration of spermidine with CH_3-G prevented these drug-related changes in spleen but not in bone marrow. By appropriate scheduling, spermidine also reversed the changes caused by CH_3-G in spleen cells.

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MATERIALS AND METHODS

Chemicals. Methylglyoxal-bis(guanylhydrazone) dihydrochloride was obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, MD. Labeled *S*-adenosyl-L-methionine [^{14}COOH] (10 mCi/mmole) was purchased from New England Nuclear and radioactive sodium chromate (^{51}Cr) was purchased from Amersham/Searle. Spermidine (grade A) was obtained as the trihydrochloride salt from CalBiochem. Both CH_3-G and spermidine were dissolved in saline immediately before injection.

Treatment of animals. Inbred C57Bl/6 Jacobs (C57Bl/6Ja) female mice, 10–12 weeks old, obtained from the animal breeding center at Roswell Park Memorial Institute, West Seneca, NY, were used throughout this study. Mice were immunized i.p. with a single dose of 5×10^8 SRBC as antigen. Treatment with CH_3-G, spermidine, CH_3-G plus spermidine, or CH_3-G followed by spermidine was begun 3 hr after the administration of SRBC. Each agent was given i.p. in a dose of 85 mg/kg/day in accordance with the schedules outlined in Results. There was no evidence of toxicity with any of the drug schedules reported in this study.

Cell suspensions. Each treatment group consisted of four mice from which spleen, femurs and tibias were removed. The spleens from each group were pooled and single cell suspensions were obtained [9]. The femurs and tibias from each group were also pooled. The ends of the bones were severed and a 25 gauge needle attached to a 3 cm^3 syringe containing RPMI 1640 medium was inserted into the marrow cavity. Marrow was flushed from the cavity with this medium, and clumps of marrow cells were dispersed by aspiration through a 22 gauge needle fitted to the same 3 cm^3 syringe. Cell suspensions obtained from spleen and bone marrow were washed once in saline and once in medium, resuspended in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, counted microscopically, and then used in the various assays. In all the treatment groups the cells were found to be more than 90 per cent viable by the trypan blue exclusion technique [10], and the total recovery of cells was similar in these groups.

Determination of S-adenosylmethionine decarboxylase activity. Approximately 1×10^8 spleen or bone marrow cells were washed and resuspended in 0.5 ml of ice-cold 25 mM Tris buffer (pH 7.5), containing 0.1 mM EDTA and 5 mM dithiothreitol and were then disrupted in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. Homogenates were centrifuged at 100,000 *g* for 60 min and supernatants were used as a source of enzyme. SAMDC activity was determined by measuring the release of $^{14}CO_2$ from SAM[^{14}COOH] as previously described [11]. The standard incubation mixture contained in a final volume of 0.2 ml: 0.5 μmole putrescine, 1 μmole dithiothreitol, 20 μmoles of sodium potassium phosphate buffer, pH 7.2, 0.04 μmole SAM[^{14}COOH] (~30,000 dis./min), and 50 μl of the cell extract. Incubations were carried out for 60 min at 37°. Enzyme activities were corrected for non-specific release of CO_2 with cell extract which had been heated at 100° for 10 min and thereafter assayed as above, and activities were expressed as nmoles CO_2 released/mg of protein/60 min. Protein concentrations were determined by the method of Lowry *et al.* [12].

Cytotoxicity assays. SRBC were labeled with ^{51}Cr as previously described [8], and the release of ^{51}Cr from SRBC, after incubation with either spleen cells or bone marrow cells, was used as a measure of cytotoxicity.

In complement-dependent cellular cytotoxicity (CDCC), various numbers of spleen or bone marrow cells were mixed with 1×10^5 ^{51}Cr-labeled SRBC in 12 × 75 mm plastic tubes (Falcon) in a volume of 0.2 ml RPMI 1640 medium containing 5% heat-inactivated fetal calf serum and incubated at 37° in an atmosphere of 10% CO_2 in air for 45 min. Guinea pig complement (0.4 ml of a 1 to 40 dilution) was then added and the incubation was continued for another 45 min. In this test, antibody to SRBC secreted from immune spleen or immune bone marrow cells binds to target SRBC and marks them for complement-mediated cytotoxicity [13]. The complement-independent cellular cytotoxicity (CICC) differed from CDCC in that the incubation of spleen or bone marrow cells with labeled SRBC was carried out for 20 hr and no complement was added. In this test the secreted antibody bound to target SRBC marks them for the lytic action of killer cells contained in the spleen or bone marrow cell populations [14].

The CDCC and CICC reactions were stopped by diluting the incubation mixtures with 2 ml of ice-cold medium. In order to determine the release of ^{51}Cr in each test, cell suspensions were centrifuged at 500 *g* for 5 min and supernatants were poured off into 12 × 75 mm disposable glass tubes. Radioactivity in both pellet and supernatant was obtained as cpm in a Packard Auto-Gamma spectrometer. The per cent of ^{51}Cr released from the SRBC target cells was calculated as a ratio in the following way:

$$\%\ ^{51}\text{Cr release} = \frac{\text{supernatant (cpm)}}{\text{pellet (cpm)} + \text{supernatant (cpm)}} \times 100.$$

The per cent specific release was the difference between the percentage ratios obtained with spleen or bone marrow cells from immunized animals and those obtained with spleen or bone marrow cells from non-immunized animals.

All assays in this investigation were carried out in duplicate and each experiment was repeated at least three times. Although there was considerable variation in the absolute values obtained from each experiment, the differences between drug-treated and control groups were consistent and did not vary by more than 15 per cent from experiment to experiment in the case of immunosuppression and by more than 40 per cent in the case of enzyme enhancement.

RESULTS

Immunosuppressive effects of CH_3-G. Daily i.p. treatment of mice, immunized against SRBC, with CH_3-G (85 mg/kg) for 5 days resulted in a marked suppression on day 7 of the CDCC and CICC immune responses of both spleen and bone marrow cells to ^{51}Cr-labeled SRBC at all effector to target cell

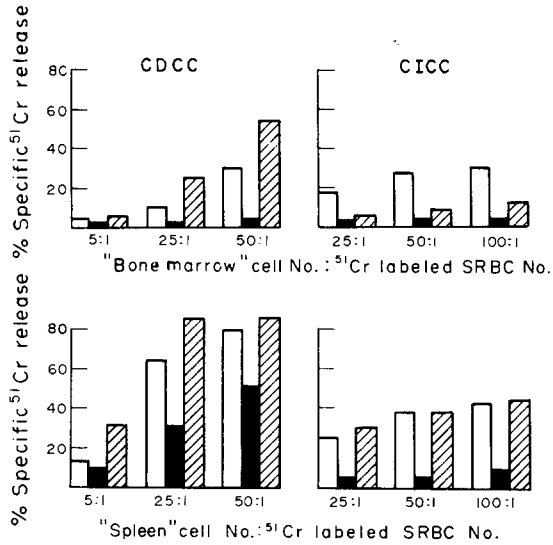

Fig. 1. Effects of CH_3-G and CH_3-G plus spermidine on CDCC and CICC responses of mouse bone marrow and spleen cells at different effector to target cell ratios. C57Bl/6Ja mice were immunized with a single i.p. injection of 5×10^8 SRBC (□) on day 0, and their bone marrow and spleen cells were tested for immune functions on day 7. Some groups of mice were also injected i.p. with an 85 mg/kg dose of CH_3-G (■), or with an 85 mg/kg dose of CH_3-G plus an 85 mg/kg dose of spermidine (■) on day 0, 3 hr after antigen administration, and again on days 1, 2, 3 and 5. Spleen or bone marrow cells were incubated with ^{51}Cr-labeled SRBC at the ratios indicated below the corresponding set of bars and assayed for CDCC and CICC as described in Materials and Methods. The CDCC and CICC responses obtained with spleen and bone marrow from mice immunized with SRBC and treated with spermidine were 2 to 7 units greater than the responses obtained in the immunized control group.

ratios studied (Fig. 1). Immunosuppression appeared to be greater in bone marrow than in spleen cells. Co-administration of equal doses of spermidine with CH_3-G resulted in a prevention of the drug-induced suppression of the CDCC and CICC responses of spleen cells and the CDCC response of bone marrow cells but not of the CICC response of bone marrow cells. Treatment with spermidine alone had no influence on the immunological responses (data not shown).

Marked inhibitions of CDCC and CICC responses in both bone marrow and spleen cells were also observed 6 days after immunization in CH_3-G-treated groups (Fig. 2). These inhibitions persisted in bone marrow cells, whereas in spleen cells a gradual recovery occurred by days 7 and 8. Co-administration of spermidine completely prevented the CH_3-G-related suppression of the CDCC and CICC responses in spleen cells at the times studied. In contrast, in bone marrow cells spermidine did not prevent the suppression of these responses on day 6; by day 7, spermidine prevented the suppression of the CDCC response and by day 8, prevention of both responses was seen (Fig. 2).

Enhancement in the activity of SAMDC after CH_3-G administration. The CH_3-G-induced changes in the activity of SAMDC and the effect of spermidine co-administration on these changes were investigated in bone marrow and spleen cells in order to determine whether drug-induced changes in enzyme activity would manifest a selectivity similar to that found in the effects on immunological function. As shown in Table 1, the activity of SAMDC was increased more than 30-fold over control values in both bone marrow and spleen cells from mice treated with CH_3-G after the 5-day drug treatment schedule outlined in Fig. 1. This increase had previously been found to be independent of immunization [8]. Administration of spermidine alone did not appear to affect the activity of

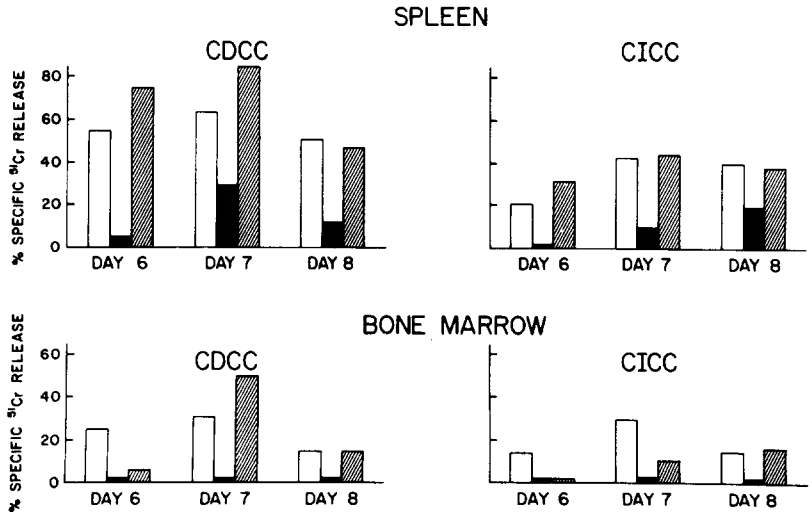

Fig. 2. Time course of immunosuppression in mouse bone marrow and spleen cells after treatment with CH_3-G, and the prevention of this suppression by co-administration of spermidine. C57Bl/6Ja mice were treated and their bone marrow and spleen cells were tested at the above indicated times as outlined in the legend for Fig. 1. The effector to target cell ratio was 10:1 for the CDCC response and 50:1 for the CICC response. The symbols indicate groups similar to those for Fig. 1.

Table 1. Activity of SAMDC in bone marrow and spleen cells from mice treated with CH_3-G, spermidine, or CH_3-G plus spermidine*

Drug treatment	CO_2(nmoles/mg protein/60 min)	
	Bone marrow	Spleen
Control	0.8	0.3
CH_3-G	27.2	12.7
Spermidine	1.2	0.2
CH_3-G + spermidine	21.9	3.8

* C57Bl/6 Ja mice were treated i.p. with CH_3-G (85 mg/kg), spermidine (85 mg/kg), or CH_3-G plus spermidine (85 mg/kg each drug) on days 0, 1, 2, 3 and 5. Mice were sacrificed on day 7 and bone marrow and spleen cells were tested for SAMDC activity as described in Materials and Methods.

SAMDC in either tissue (Table 1). However, after the co-administration of spermidine, the CH_3-G-mediated increase in SAMDC activity in spleen was only 33 per cent whereas the increase in bone marrow was nearly 80 per cent of that found with CH_3-G alone (Table 1).

Time-dependent kinetics of SAMDC activity was studied after a single dose of CH_3-G or CH_3-G plus spermidine. The maximal increase in enzyme activity was 20- to 40-fold at 48 hr after CH_3-G in both spleen and bone marrow, although the basal level of enzyme activity was consistently higher in the bone marrow. In agreement with previous results, there was a 50 per cent reduction in such increase of enzyme activity in the spleen after spermidine co-administration, whereas there was only a slight reduction of that increase in the bone marrow (Table 2).

Reversal of CH_3-G effects by spermidine. In order to explore the possibility that spermidine may also antagonize CH_3-G at intracellular sites, spermidine was administered, once daily, i.p., for 3 days. The first spermidine injection was given 24 hr after the final i.p. treatment with CH_3-G, which was administered once daily on days 0 and 1 or on days 0, 1 and 2. As shown in Fig. 3, administration of spermidine on days 2, 3 and 4 reversed the immunosuppression

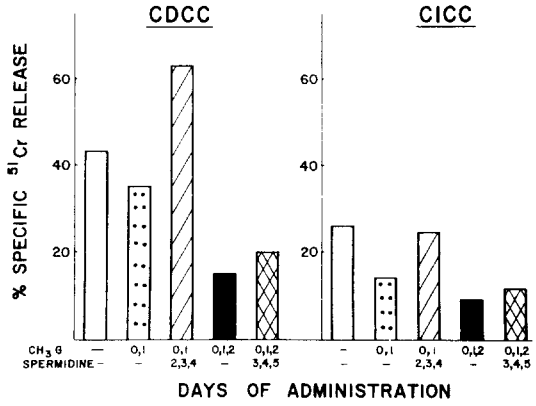

Fig. 3. Effect of administration of spermidine subsequent to that of CH_3-G on the immunosuppressive action of CH_3-G. C57Bl/6Ja mice were immunized with a single i.p. injection of 5×10^8 SRBC on day 0 and were then treated with CH_3-G or CH_3-G followed by spermidine on the days indicated in the figure. Spleen cells were harvested on day 7 and were incubated with ^{51}Cr-labeled SRBC at an effector to target cell ratio of 10:1 for 1.5 hr in the CDCC test and at a ratio of 50:1 for 20 hr in the CICC test.

resulting from CH_3-G treatments on days 0 and 1. Similarly, such a schedule of spermidine administration also resulted in a reduction of accumulation of SAMDC to about 33 per cent of that seen with CH_3-G alone (Table 3). However, when treatment with CH_3-G was prolonged for 1 more day, no significant reversal of immunosuppression occurred by administering spermidine for the next 3 days (Fig. 3) although the activity of SAMDC was reduced to about 50 per cent of that seen with CH_3-G alone (Table 3).

DISCUSSION

CH_3-G treatment resulted in suppression of the function of immune effector cells from both bone marrow and spleen. However, this drug effect appeared to be greater in the bone marrow. The enhancement of SAMDC activity was also greater in the bone marrow, suggesting a possible parallel

Table 2. Kinetics of enhancement of SAMDC in bone marrow and spleen from mice treated with CH_3-G or CH_3-G plus spermidine*

Drug treatment	CO_2(nmoles/mg protein/60 min)					
	Bone marrow			Spleen		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Control	0.7	0.5	0.8	0.2	0.3	0.3
CH_3-G	10.3	20.1	5.9	4.1	6.0	2.5
CH_3-G + spermidine	9.8	19.4	5.0	1.8	3.5	1.8

* C57Bl/6Ja mice were injected i.p. with a single 85 mg/kg dose of CH_3-G or CH_3-G and 85 mg/kg of spermidine. At the indicated times after drug administration, mice were sacrificed, and the activity of SAMDC was determined in cells from bone marrow and spleen as described in Materials and Methods. Tissues from mice receiving spermidine alone possessed the enzyme activities within $\pm$ 0.2 units of the control.

Table 3. Effect of administration of spermidine subsequent to that of CH_3-G on the CH_3-G-mediated enhancement of SAMDC in mouse spleen cells*

Treatment		
Drug(s)	Days of Adm.	CO_2(nmole/mg protein/60 min)
Control		0.1
CH_3-G	0, 1	1.4
CH_3-G + spermidine	0, 1 2, 3, 4	0.4
CH_3-G	0, 1, 2	1.2
CH_3-G + spermidine	0, 1, 2 3, 4, 5	0.6

* C57B1/6Ja mice were treated with CH_3-G or CH_3-G followed by spermidine on the days indicated in the table. Spleen cells were harvested on day 7, and the activity of SAMDC was determined as described in Materials and Methods.

between the effect on enzyme and immunosuppression. The biochemical basis for the accumulation of SAMDC in cells treated with CH_3-G is believed to be due to stabilization of the enzyme rather than to increased synthesis [5]. The basis for immunosuppression by CH_3-G is not clear. It may result from either an inhibition of spermidine and spermine biosynthesis [15, 16] or from a direct interaction of CH_3-G with some macromolecules [17, 18] in cytoplasm or within some organelle, such as, mitochondria [19]. Indeed, mitochondria seem to be a selective target site for CH_3-G action in L1210 cells [20]. Since the blood flow to spleen does not appear to be slower than that to the bone marrow, the greater effects of CH_3-G in bone marrow may be due to a greater uptake and/or retention of CH_3-G by bone marrow cells.

Co-administration of spermidine with CH_3-G was more effective in preventing immunosuppression of spleen cells than of bone marrow cells. This effect together with the greater reduction in the accumulation of SAMDC in spleen cells may be related to a differential distribution of spermidine in the two tissues which could result in a greater inhibition of the CH_3-G uptake at the plasma membrane level in spleen cells [6]. However, since administration of spermidine subsequent to CH_3-G under the appropriate schedule was indeed effective in reversing the drug effect, the basis for such antagonism may involve intracellular sites as well. It is known that CH_3-G causes a specific inhibition of the putrescine-activated SAMDC, this resulting in the inhibition of spermidine and spermine biosynthesis [16, 21]. Although the enzyme protein accumulates in the presence of CH_3-G, possibly due to stabilization [5], the enzyme activity in CH_3-G-treated cells does not seem to be adequate to maintain spermidine pools [16]. When the drug concentration is reduced by dilution or dialysis, the enzyme activity is measurable and is considerably higher than that of untreated controls. If the cytotoxicity of CH_3-G were indeed related to its ability to inhibit polyamine biosynthesis, this would be consistent with the observed correlation between drug action on SAMDC accumulation and immunosuppression. Alternately, inhibition of polyamine biosynthesis and accumulation of SAMDC may represent independent drug effects occurring in parallel with the immunosuppressive effect of CH_3-G. The above correlation between increased accumulation of SAMDC and decreased cellular function, although qualitative in nature, may be useful in obtaining a gross assessment of the sensitivity of a target cell type to CH_3-G. As such it may be helpful in improving the therapeutic application on this agent. Thus, further work is indicated to verify this possibility.

It has been shown previously that co-administration of spermidine antagonizes the antiproliferative effects of CH_3-G [3]. However, the possibility that delayed administration of spermidine may actually reverse CH_3-G action has not been explored previously. Our data (Table 3 and Fig. 3) not only show that such a reversal or rescue occurs but also that a quantitative difference exists in this respect between the two tissues examined. Regardless of its basis, it may be worthwhile to explore further the rescue from CH_3-G action by spermidine with a view to increase the selectivity of anti-leukemic action of CH_3-G by appropriate sequential scheduling of CH_3-G and spermidine.

REFERENCES

1. V. T. Oliverio and C. Zubrod, *A. Rev. Pharmac.* **5**, 335 (1965).
2. R. H. Levin, E. Henderson, M. Karon and E. J. Freireich, *Clin. Pharmac. Ther.* **6**, 31 (1965).
3. E. Mihich, in *Antineoplastic and Immunosuppressive Agents* (Eds. A. Sartorelli and D. Johns), pp. 358–70. MacMillan, NY (1973).
4. H. G. Williams-Ashman and A. Schenone, *Biochem. biophys. Res. Commun.* **46**, 288 (1972).
5. A. E. Pegg, A. Corti and H. G. Williams-Ashman, *Biochem. biophys. Res. Commun.* **52**, 696 (1973).
6. C. Dave and L. Caballes, *Fedn Proc.* **32**, 736 (1973).
7. D. H. Russell, *Life Sci.* **13**, 1635 (1973).
8. J. Bennett, J. Ehrke, C. Dave and E. Mihich, *Biochem. Pharmac.* **26**, 723 (1977).
9. S. A. Cohen, M. J. Ehrke and E. Mihich, *J. Immun.* **115**, 1007 (1975).
10. D. J. Merchant, R. H. Kahn and W. H. Murphy, in *Handbook of Cell and Organ Culture*. Burgess Publishing Co., Minneapolis.
11. A. E. Pegg and H. G. Williams-Ashman, *J. biol. Chem.* **244**, 682 (1969).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall *J. biol. Chem.* **193**, 265 (1951).
13. C. Mawas, T. Carey and E. Mihich, *Cell. Immun.* **6**, 243 (1973).

14. P. Perlmann and H. Perlmann, *Cell Immun.* **1**, 300 (1970).
15. D. H. Russell and O. Heby, *Cancer Res.* **34**, 886 (1974).
16. A. E. Pegg, *Biochem. J.* **132**, 537 (1973).
17. E. Mihich, *Cancer Res.* **23**, 1375 (1963).
18. A. C. Sartorelli, A. T. Iannotti, B. A. Booth, F. H. Schneider, J. R. Bertino and D. G. Johns, *Biochim. biophys. Acta* **103**, 174 (1965).
19. C. Dave, J. Ehrke and E. Mihich, *Chem. Biol. Interact.* **12**, 183 (1976).
20. S. N. Pathak, C. W. Porter and C. Dave, *Cancer Res.* **37**, 2246 (1977).
21. A. Corti, C. Dave, H. G. Williams-Ashman, E. Mihich and A. Schenone, *Biochem. J.* **139**, 351 (1974).