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INHIBITION OF LYMPHOCYTE DNA-SYNTHETIC RESPONSES BY SPERMINE-DERIVED POLYCATIONS*

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Abstract—The polyamines putrescine, spermidine, and spermine are oxidized by the enzyme diamine oxidase to form the corresponding aldehyde derivatives. These aldehydes have been shown to undergo a variety of spontaneous reactions, some of which result in polycationic addition compounds. We have chemically synthesized some spermine-derived polycations by reaction with the dialdehyde glutaraldehyde followed by reduction of the resulting Schiff base with sodium borohydride. Their migration on ion exchange and gel filtration columns was consistent with the formation of polycations with properties similar to those reported for the spontaneous reaction products. When added to cultures of alloantigen or mitogen stimulated lymphocytes, these polycations were potent inhibitors of the incorporation of tritiated thymidine and blast cell formation. This inhibition was reversible, non-cytotoxic, and only apparent if the polycation was added early in the culture period. The concentration of polycation necessary to achieve 50% inhibition of the lymphocyte response decreased as the cationic nature relative to spermine increased.

The polyamines spermine and spermidine are universal components of animal cells and body fluids. The biosynthesis, distribution, and certain biological effects of polyamines have been reviewed extensively [1, 2]. In general, increases in polyamine levels and the associated synthetic and degradative enzymes are correlated with increased cellular proliferation [2, 3]. The polyamines are substrates for diamine oxidase [amine:oxygen oxidoreductase (deaminating) (pyridoxal containing) EC 1.4.3.6] which catalyzes the oxidation of the primary amino groups to aldehydes. High levels of diamine oxidase (DAO) have been found in certain tumors [4, 5], transformed cells [6], and regenerating tissues [7, 8]. It is also present in higher than normal levels in human serum during pregnancy [9]. This increase corresponds with the increased levels of the polyamines which are considered essential for cellular growth [2].

The products of DAO (the oxidized polyamines) have been found to undergo a variety of spontaneous reactions. Oxidized spermine has been shown to form the toxic aldehyde acrolein [10, 11]. Putrescine is oxidized to γ-aminobutyraldehyde which can spontaneously cyclize to form Δ^1-pyroline [12]. Dioxidized spermine has also been shown to form uncharacterized polycations with net charge greater than the starting compound [13]. It is this last reaction which is of particular interest to us. High levels of DAO have been found in human seminal fluid along with high levels of polyamines [14, 15]. The K_m for spermine of the seminal plasma DAO is about 0.1 mM [16], while the concentration of spermine is about 3 mM in the same fluid. Under these circumstances, the presence of some condensation products would appear likely. Cystic fibrosis patients also have detectable levels of DAO in saliva which is capable of forming oxidation and condensation products with endogeneous polyamine. These condensation products were many times more active than the unreacted spermine in the inhibition of sodium transport [17].

The addition of spermine and spermidine to culture media has been inhibitory to a number of cellular responses *in vitro* [18, 19]. In general, it has been found that inhibition of cell proliferation by polyamines has been apparent only if the cells were grown in the presence of fetal calf serum. Polyamines were not inhibitory in the presence of human or other non-ruminant sera. In all cases the inhibition has been correlated with the presence of diamine oxidase in the serum. Addition of DAO to culture media containing human serum and polyamines will result in growth inhibition. It has been assumed that the formation of acrolein from polyamine aldehydes is the active agent responsible for the growth inhibition observed by polyamines in the presence of DAO. In fact, inhibitors of DAO will prevent the growth inhibition usually observed with polyamines; thus, the enzymatic oxidation of spermine and spermidine would appear to be necessary for the growth inhibitory effects to be apparent with cultured cells. As mentioned above, however, other products, in addition to acrolein, are also possible.

In these preliminary studies, we set out to determine if polyamine condensation products could be synthesized by chemical means and to measure the biological and biochemical properties of these compounds relative to spermine. We have synthesized some polycations from spermine by polymerization with glutaraldehyde, a dialdehyde similar in structure to dioxidized spermine. These polycations were many times more active than the parent compound in several *in vitro* models of lymphocyte DNA-synthetic responses. This is similar to the effects seen

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by Dearborn [17] with naturally formed polycations from CF patient saliva or seminal fluid and sodium transport inhibition. The study of these polyamine condensation products may provide an explanation for the observed inhibition by polyamines in the presence of fetal calf serum or DAO on cell proliferation and provide a route to new polyamine analogues useful in studying polyamine function. In addition, these model compounds will prove useful in studying the possible effects of such polycations on other biological systems, as these compounds may have some different effects than polycations such as poly-L-lysine or compound 48/80. Such polyamine derivatives should have extremely interesting and unusual biological effects.

MATERIALS AND METHODS

Synthesis. For a typical preparation, 1.4 mmoles of spermine tetrahydrochloride was dissolved in 5 ml of distilled water and the pH was adjusted to 7–8 by addition of small amounts of sodium carbonate. To this rapidly stirring solution was added 0.25 mmole of freshly diluted glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in several small aliquots, and the reaction mixture was left overnight at 4°. The resulting solution was then adjusted to pH 8.5 to 9 with more solid sodium carbonate and reduced by the addition of a 5- to 10-fold molar excess of freshly prepared sodium borohydride ($NaBH_4$) in distilled water adjusted to pH 8–9 as above. After reduction was complete (overnight at 4° in the dark), the reaction was acidified by addition of glacial acetic acid and dried under reduced pressure. The residue was then redissolved in 20 ml of 1 mM HCl and relyophilized. This procedure was repeated twice.

In one preparation, a purified product from a reaction performed as described above was reacted on a small scale. An aliquot of SP-Sephadex pool III (see text) containing an estimated 23 μmoles of product was reacted with 250 μmoles of glutaraldehyde in a final volume of 0.6 ml as above. Reduction was performed as described above. The total reaction scheme was performed in polystyrene plastic tubes.

Ion-exchange chromatography. After lyophilization, the reaction products were separated on a 1.5 × 40 cm column of SP-Sephadex S-25 prepared as described [13, 17]. The column was washed and equilibrated in distilled water. The sample was dissolved in sufficient distilled water to give a conductivity of between 2000 and 4000 μmhos and centrifuged to remove any insoluble material. The sample was applied and the column was washed with distilled water until the absorbance at 220 nm returned to baseline. At least 5 column volumes were washed through the column. The column was then eluted with a gradient of distilled water to 0.25 M HCl (100 ml each) followed by a gradient of 0.25 M HCl to 3 M HCl (250 ml each). Fractions of 10 ml were collected.

For small scale reactions, columns containing 5 ml of gel were prepared in plastic syringes fitted with Luer Lok plastic valves. The sample was diluted to low conductivity as above and the column was eluted batchwise with 25 ml fractions of distilled water, 0.1 N HCl, 1 N HCl, 1.5 N HCl, 2 N HCl, and 3 N HCl. Each fraction was lyophilized twice to remove HCl and assayed by ninhydrin and high voltage (H.V.) paper electrophoresis as described below.

Gel filtration. Gel filtration of polyamine fractions was performed on a 2.2 × 90 cm column of Sephadex G-25 equilibrated with 0.1 M pyridine acetate, pH 4.5. Fractions of 10 ml were collected. The column was calibrated with bacitracin (mol. wt 1411), oxidized glutathione (612) and reduced glutathione (307). Standards were detected by their absorbance at 280 nm or by reaction with ninhydrin.

High voltage electrophoresis. High voltage paper electrophoresis was performed on a flat bed apparatus on Whatman 3 MM paper. Samples were spotted and dried on an origin near the cathode end of the paper strip. The paper was moistened in 0.5 M sodium acetate buffer, pH 5.0, and the electrode chambers contained the same buffer. This high ionic strength is necessary to avoid streaking of the highly cationic polyamines. Electrophoresis was performed at 800 V per 14 cm wide strip for 120 min. After electrophoresis, the papers were dried at 70°.

Ninhydrin reactions. Dried paper from H.V. electrophoresis was dipped in a solution of 1 g ninhydrin dissolved in acetone–acetic acid (80:20) containing 100 mg cadmium acetate. The paper was air dried and heated at 70° until characteristic spots developed.

For screening of column fractions or assay of pooled material and standards the following procedure was used. First, aliquots of the fractions and standards were lyophilized to remove solvents and volatile buffers. To these tubes was added 1 ml of ninhydrin reagent stock solution [400 mg $SnCl_2 \cdot 2H_2O$ in 250 ml of 0.2 M citrate buffer, pH 5.0 mixed with 250 ml of methyl Cellosolve (Union Carbide Corp.) containing 10 g ninhydrin]. The tubes were mixed and heated for 20 min in a boiling water bath. After cooling, a sufficient volume of 50% isopropanol was added such that the most concentrated sample did not exceed two absorbance units at 570 nm. The resulting solutions were read at 570 nm relative to a solvent bank.

Biological assays. For the mixed lymphocyte culture (MLC) assay, the spleens were removed from C57/BL and Balb/c mice and each was placed in a Petri dish containing cell collection medium (Earl's Balanced Salt solution containing 2.5% heat-inactivated fetal calf or human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 I.U./ml heparin). The spleen was perfused briefly using a 22- or 25-gauge needle to remove most red blood cells, placed in a second dish, and teased gently to remove the spleen lymphocytes. The large pieces of tissue were removed by passage through a 50-mesh screen, and the lymphocytes were recovered by centrifugation at 200 *g* for 10 min. The cells were washed once with cell collecting medium and resuspended in complete assay medium, RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal calf or human serum, 5×10^{-4} M mercaptoethanol, and 20 mM HEPES* buffer. The MLC was set up by adding

* HEPES, 4-(2-hydropyethyl)-1-piperazine-ethanesulfonic acid.

1.25×10^5 cells/well from each strain to microwell plates. The plates were incubated at 37° in a humidified atmosphere containing 5% CO_2 for 4 days. Tritiated thymidine (0.5 μCi, 2 Ci/mmole) was added for the last 6 hr of the culture period. At the end of the culture period, the extent of inhibition of large blast cell formation was estimated by microscopic examination of the plates. The cells were harvested using a Skatron cell harvester, and the amount of radioactive label incorporated was determined after addition of 0.5 ml of Soluene tissue solubilizer and 4 ml of Dimilume-30 scintillation fluid (Packard Instrument Co., Downers Grove, IL).

Mitogen stimulation of Balb/c spleen lymphocytes was carried out with experimentally determined optimum levels of mitogens. These were: Concanavalin A (Con A, DifCo Laboratories, Detroit, MI) at 0.5 μg per 250,000 spleen cells per 0.2 ml medium and lipopolysaccharide (LPS) from *Salmonella typhosa* (Sigma Chemical Co., L-3630) at 1.5 μg per 250,000 spleen cells per 0.2 ml medium. Cultures with Con A and LPS were incubated for 72 hr, after which the cells were examined and labeled as for the MLC.

Suspensions of spontaneously dividing Balb/c and C57/BL spleen cells and thymocytes were prepared essentially as were the cells to be used for MLC. The cell suspension, at 5×10^5 cells per well, was incubated for 24 hr with the test samples and then was labeled and harvested as described for the MLC.

Dose curves were calculated for both visual and [^{3}H]thymidine inhibition, and a 50% inhibitory dose (ID_{50}) was calculated for a particular sample and expressed as μg per ml concentration necessary for 50% inhibition.

RESULTS

In these preliminary studies we have synthesized oligomeric forms of the polyamine spermine by reaction with glutaraldehyde. The reaction scheme involves the reaction of spermine with the dialdehyde to form the Schiff base and reduction of the C=N bonds by $NaBH_4$. This reaction mechanism and procedure have been adapted from those commonly used for the reductive methylation of protein amino groups with formaldehyde [20, 21] and for the formation of pyridoxal polyamine compounds [22]. Initial small scale reactions showed that the reaction conditions described under Materials and Methods gave rise to ninhydrin reactive species which migrated, in H.V. paper electrophoresis, more slowly than spermine. They also showed considerable streaking even in 0.5 M buffer. We have found that standard spermine will migrate as a distinct spot only in the 0.5 M buffer, due to, we presume, interactions of this highly cationic compound with the paper.

The reaction products were separated by chromatography on SP-Sephadex S-25, using a gradient of HCl. The elution profile is shown as Fig. 1. There are two small peaks of ninhydrin positive material that eluted after spermine at concentrations of greater than 1 M HCl. These compounds, therefore, had charges greater than +4 under these conditions. The indicated pools were made based on the ninhydrin reactive peaks, lyophilized, and analyzed by high voltage paper electrophoresis. These results are shown diagrammatically in Fig. 2. Two fractions, III and IV, migrated more slowly than spermine as might be expected from the calculated charge/mass ratios for expected reaction products and from our results with other diamines and polyamines. Also,

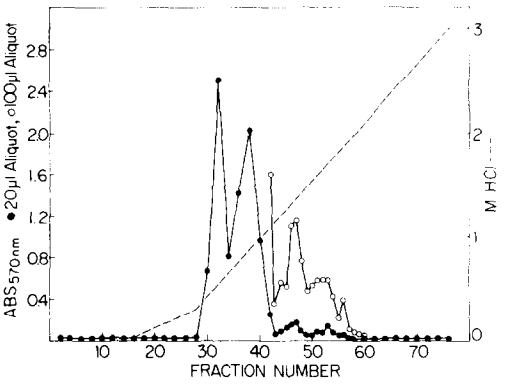

Fig. 1. Elution profile of the reaction products from a 1.5×40 cm column of SP-Sephadex. The sample was applied in distilled water and the column was washed with five column volumes of distilled water before the start of the HCl gradients. For analysis, either 20 μl or 100 μl aliquots of the fractions were taken, lyophilized to dryness to remove HCl, and reacted with ninhydrin. The absorbance at 570 nm was then determined. Pools of active material were made as follows: pool I, tubes 29–34; pool II, tubes 35–42; pool III, tubes 44–49; and pool IV, tubes 50–58. These pools were lyophilized several times to remove traces of HCl.

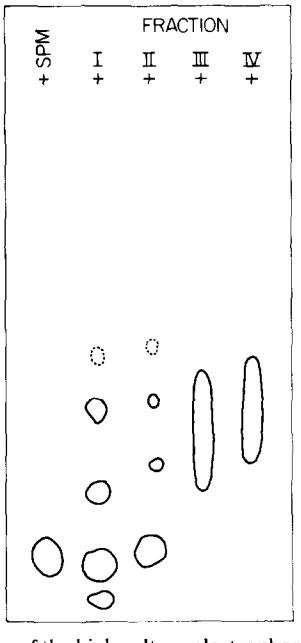

Fig. 2. Diagrams of the high voltage electrophoresis pattern obtained by ninhydrin stain. Aliquots of the indicated pools from the column shown in Fig. 1 were electrophoresed on Whatman 3 MM paper in 0.5 M sodium acetate, pH 5.5, at 800 V for 90 min. The paper was dried and spots were developed by dipping in standard ninhydrin–Cd reagent and drying at 70°. SPM designates standard spermine.

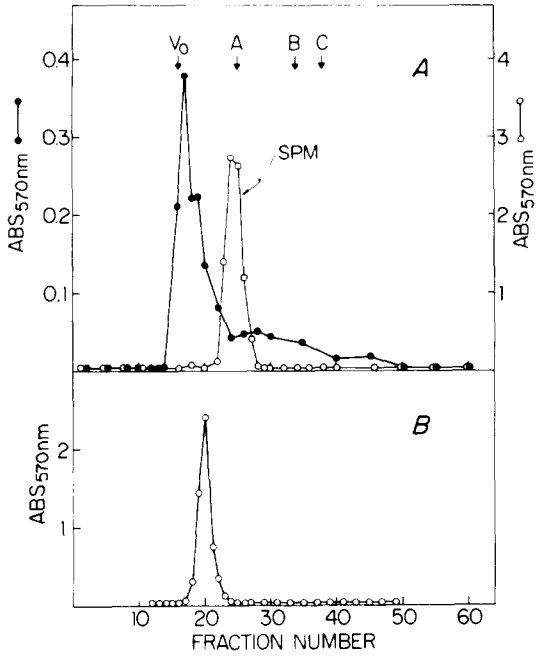

Fig. 3. (A) Sephadex G-25 elution profile of standard spermine (SPM) and SP-Sephadex pool IV. (B) Elution profile of SP-Sephadex pool III. Column conditions and analysis by ninhydrin reactions were as described in Materials and Methods.

there was considerable streaking of these compounds which is also an indication of the highly charged nature of these fractions. These fractions were eluted from the SP-Sephadex column at a HCl concentration similar to that reported for addition products of isolated dioxidized spermine [13] and for the products of seminal fluid or cystic fibrosis saliva DAO [17].

We have shown [23] that spermine migrated in an anomalous manner on Sephadex G-25 columns, suggesting a molecular weight of 1400 rather than the true weight of 202. Similar results have been reported for spermidine [24]. We have found that Sephadex G-25 can still be used to estimate the molecular weights of the reaction products, however. The elution profiles of standard spermine and the two SP-Sephadex fractions are shown in Fig. 3 (A and B). The two fractions migrated clearly ahead of spermine on the G-25 indicating higher apparent molecular weights. Since these polyamines migrated differently from peptides on this column, we have used the following method to estimate molecular weights. The column was calibrated with peptide standards and with spermine which eluted with the bacitracin marker at an apparent molecular weight of 1400. A standard plot of log molecular weight versus elution volume was drawn, and then a parallel line was drawn, offset enough to give the spermine its true molecular weight of 202. Using this calibration line, the molecular weights of fractions III and IV were determined to be 490 and 740 respectively. These results are consistent with Fraction III being a dimer and Fraction IV being a trimer of spermine linked via reduced glutaraldehyde groups. We have not performed more detailed analysis of the structures of these polycations due to the limited amounts available presently. The preliminary characterization by ion exchange and gel filtration chromatography and by H.V. paper electrophoresis suggests that dimers and trimers of spermine were formed and that these polycations were similar to the spontaneous reaction products.

We have re-reacted an aliquot from the SP-Sephadex Fraction III and separated the products on a small SP-Sephadex column. Analysis of this sample by H.V. paper electrophoresis indicated the formation of a possible tetrameric form of spermine. We do not presently have sufficient material for a molecular weight estimate.

The yields for these reaction products for a typical synthesis, assuming the molecular weight from G-25, have only been an estimated 3–5%. While the reaction conditions were probably not optimal, these conditions yielded sufficient material to perform preliminary biological studies which were necessary to determine if these compounds had activity related to the natural reaction products of oxidized polyamines.

As our primary biological assay for these initial studies, we have used the inhibition of the two-way

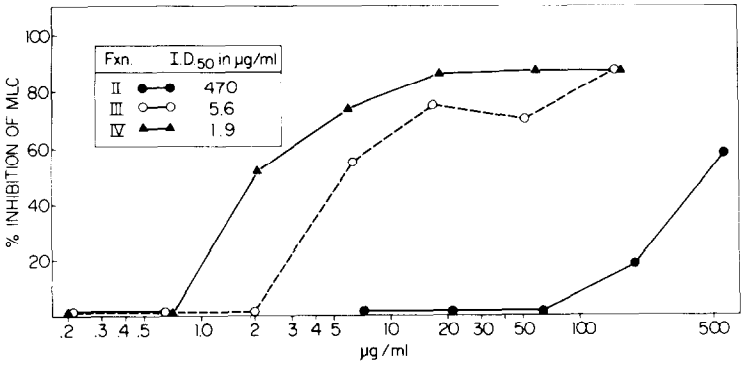

Fig. 4. Dose curves in MLC for pools II, III, and IV from the SP-Sephadex column. Solutions of the pools were made up at 5 mg/ml in 20 mM acetic acid, sterilized by millipore filtration, and assayed for the ability to inhibit MLC. The inhibition shown is for [^{3}H]thymidine incorporation; microscopic examination showed similar results.

Table 1. Effect of serum on the MLC inhibiting activity of spermine and spermine-derived polycations*

Compound	ID_{50} for MLC* (μg/ml) FCS	Human serum
Spermine	4.5	200
SP-Seph Fxn II	15	83
SP-Seph Fxn III	13	13.5
SP-Seph Fxn IV	10.5	9.0

* The 50% inhibitory dose was calculated from dose curves set up in mixed lymphocyte cultures run either in FCS or human serum and assayed for [^{3}H]thymidine incorporation as described under Materials and Methods. Microscopic estimation of blast cell formation was in agreement with the [^{3}H]thymidine dose curves.

mixed lymphocyte culture (MLC). This assay involved the alloantigenic stimulation of spleen cells from two strains of mice (C57 and Balb/c) to undergo blast transformation. This assay was extremely useful, as the extent of reaction can be determined by microscopic examination of the cultures and estimation of the extent of blast cell formation and can be quantitated by determining the incorporation of [^{3}H]thymidine. Using the assay, we have determined the inhibitory ability of the fractions from the SP-Sephadex column. The dose curve for the inhibition of [^{3}H]thymidine incorporation in an MLC run in human serum is shown in Fig. 4. As can be seen, fraction II, which by electrophoresis was mainly spermine, was the least active inhibitor, while the two fractions that eluted at higher HCl concentrations (Fractions III and IV) were increasingly more active in the order of their elution from the SP-Sephadex column. It would appear, therefore, that increasing polycationic nature (as measured by elution from SP-Sephadex) was associated with increasing inhibition of the MLC. This inhibitory activity was independent of the serum used to culture the cells, in contrast to spermine which was much more active in fetal calf serum (FCS) than in human serum. This is demonstrated by the results shown in Table 1. The increased inhibitory activity of both spermine and the spermine-rich Fxn II presumably resulted from the action of diamine oxidase present in FCS but not in human serum. Fractions III and IV were equally active in either type of serum.

Table 2. Inhibition of mixed lymphocyte cultures by spermine and spermine-derived polycations*

Compound	R_{spm}	ID_{50} (μg/ml ± S.D.)
Spermine	1.0	335 ± 190
Fraction III	0.68–0.86	3.5 ± 2.8
Fraction IV	0.61–0.81	2.1 ± 0.6
Fraction 1.5 N	0.44–0.69	1.5 ± 0.6

*The 50% inhibitory dose, ID_{50}, was calculated from dose curves of the indicated fractions assayed in MLC in human serum and assayed for [^{3}H]thymidine incorporation as described under Materials and Methods. Microscopic estimation of blast cell formation was in agreement with the [^{3}H]thymidine dose curves. Fraction III was assayed in five experiments, Fraction IV in ten experiments, Fraction 1.5 N in three experiments, and spermine in four experiments. The data shown are the average ± the standard deviation. The migration relative to spermine, R_{spm}, was determined for several preparations in H.V. paper electrophoresis.

We have repeatedly assayed the MLC inhibition of these compounds relative to spermine and found that the inhibition was always correlated with increasing polycationic nature of the compounds. In this series of experiments, a product of re-reacted Fxn III, purified on a small scale ion exchange column designated Fxn 1.5 N, was also used. This Fxn 1.5 N has an R_{spm} in H.V. electrophoresis of 0.56 compared to 0.75 for the starting Fxn III, indicating the formation of a more cationic structure. Table 2 shows the ID_{50} dose for MLC compared to the cationic nature of the fractions as measured by their migration relative to spermine in H.V. paper electrophoresis. There is some variation in the absolute values due to the fact that the MLC assays were run in non-pooled human serum, but the correlation between increasing inhibition and polycationic nature is obvious. This is even more prominent if the increasing apparent molecular weights are considered.

The inhibitory effects of SP-Sephadex Fraction IV have been examined in detail to determine several additional parameters. The inhibition by the polyamine derivative was most pronounced when added early in the MLC, as shown in Table 3, and was reversible if washed from the cells after 24 hr of incubation (Table 4). These results indicated that

Table 3. Effect of the time of addition to MLC on the inhibition by SP-Seph Fxn IV*

Sample	Time of addition	[^{3}H]Thymidine (cpm/well ± S.D.; N = 6)	% Inhibition
Control MLC		98,200 ± 11,900	0
SP-Seph Fxn IV	0	13,700 ± 2,600	86
	+24 hr	21,900 ± 6,100	78
	+48 hr	54,100 ± 1,600	45
	+72 hr	87,300 ± 3,000	11

* Standard MLC assays were set up and the polycation was added at the indicated time after initiation of the culture. The cells were labeled and harvested 24 hr after the last addition. The SP-Sephadex Fxn IV was added at a final concentration of 3.1 μg/ml.

Table 4. Reversibility of the inhibitory effect on MLC of SP-Seph Fxn IV*

Concentration (μg/ml)	[^{3}H]thymidine (cpm/well ± S.D.; N = 6) Not washed	Washed
Control MLC	33,800 ± 1,200	29,500 ± 800
Fxn IV, 1	30,400 ± 1,300 (−15)	34,700 ± 1,200 (+17)
2.8	19,400 ± 1,500 (−46)	28,200 ± 1,900 (−5)
4.7	5,600 ± 300 (−84)	18,600 ± 1,800 (−37)

* Reversibility was determined by incubating 1×10^6 spleen cells of each type in 1.6 ml standard culture medium plus or minus the indicated concentration of polycation in 3 ml plastic culture tubes. After 24 hr, all the tubes were centrifuged at 200 *g* for 10 min to pellet the cells, and the medium was aspirated from one set of tubes and replaced with an equal volume of prewarmed medium without polycations. These tubes were centrifuged and the medium was replaced again. After this procedure, the tubes were returned to the incubator for an additional 72 hr. At the end of the incubation, the cells were resuspended and 0.2 ml aliquots were placed in microtiter wells for microscopic examination, labeling, and harvesting by the usual procedure. The numbers in parentheses are the percent inhibition relative to the appropriate control culture.

the compounds were not grossly cytotoxic as the cells were fully able to respond to MLC after washing. In addition, the uptake of the vital dye trypan blue was not significantly different between control and inhibited cultures.

The activity of this fraction has also been tested against other cells of the immune system. Several experiments utilized spontaneous [^{3}H]thymidine incorporation by suspensions of unstimulated mouse spleen cells or thymocytes. The spontaneously dividing thymocytes and spleen cells were relatively resistant to the inhibitory action of the polycation, and the thymocytes showed stimulation at the lower dose levels. Similar resistance was found when Fraction IV was added to the MLC at +72 hr. Since this was a population of cells already committed to DNA synthesis, similar to the thymocytes and unstimulated spleen cells, the results also argue against a general cytotoxic effect of the polyamine derivatives.

Fraction IV has also demonstrated inhibitory activity against Con A stimulated spleen cells with an ID_{50} of 3.1 μg/ml, and against lipopolysaccharide stimulated spleen cells with an ID_{50} of 3.8 μg/ml, similar to that found with the MLC. The inhibition is thus not apparently specific for the manner in which the DNA synthesis is stimulated. In all cases, microscopic examination for the extent of blast cell formation was consistent with the data obtained with isotope incorporation.

DISCUSSION

We originally became interested in the products of oxidized polyamines because of the potent inhibition of cell proliferation by polyamines in the presence of serum containing diamine oxidase activity. In general, it has been found that the polyamines spermine and spermidine are inhibitory to cultured cells in the presence of fetal calf serum or the enzyme diamine oxidase [18, 19, 25, 26]. Polyamines were not inhibitory in the presence of human or other non-ruminant sera which normally did not contain detectable levels of the enzyme. The amine oxidases catalyze the oxidation of the amino propyl moiety of spermine or spermidine to the aldehyde. The amine aldehydes, and the resulting breakdown product acrolein (H_2C=bCO—CHO), have been shown to be toxic to a variety of cells [10, 11]. It was assumed that the formation of acrolein from polyamine aldehydes was the active agent responsible for the growth inhibition observed by polyamines in the presence of DAO. The addition of dioxidized spermine [27] or stabilized derivatives [28] has been shown to be inhibitory in the absence of DAO. We have shown here that the activity of the spermine-derived polycations was also independent of diamine oxidase activity. Under certain conditions, the products of DAO, amino aldehydes, can form complex self-condensation products to form larger polycationic molecules. Kimes and Morris [13] have prepared dioxidized spermine and observed formation of condensation products having net charge greater than the parent compound as judged by elution from SE-Sephadex. They suggested that reaction could occur via Aldol condensation of the dioxidized spermine in a manner similar to that previously observed for glutaraldehyde [29] to yield a mixed population of products. In structure, the dioxidized spermine is similar to glutaraldehyde. In addition to Aldol condensations, reactive aldehydes could react with primary amines of unreacted polyamine to form linear, branched, or cyclic condensation products via Schiff base formation.

In an interesting study by Dearborn [17], it was shown that condensation products would form by incubation of labeled spermine with seminal fluid DAO. After a 3-hr incubation, the resulting condensation products were stabilized by reduction with $NaBH_4$ and separated by SP-Sephadex chromatography. Similar polycations were formed by incubation with cystic fibrosis patient saliva and these polycations were shown to be probable products of aldol or Schiff base formation by their base sensitivity and reducibility with labeled $NaBH_4$. Of particular interest to this discussion was the biological activity of these spermine-derived polycations. Using sodium transport as a biological assay, the polycations were effective inhibitors at about 1.0 to 375 μM primary

amine. Spermine had no effect at concentrations up to 100,000 times that of the polycations. The polycations were also 60 times more effective inhibitors than poly-L-lysine in the same assay. These uncharacterized, naturally occurring spermine-derived polycations showed a trend of increasing activity with increasing polymerization or charge relative to spermine.

The inhibitory effect of the chemically synthesized spermine-derived polycations on lymphocyte DNA-synthetic responses showed that the inhibitory activity increases as the degree of polymerization (or charge relative to spermine) increases. This trend was in many ways similar to that seen for other polycationic compounds. For example, increasing the molecular weight of polylysine resulted in increased antineoplastic activity against Ehrlich ascites cells [30], increased toxicity in mice [30], and increased leakage of potassium and other small molecules from cells [31, 32]. The polycationic compound 48/80 showed similar effects in that only the larger polymers in the mixture up to the hexamer were active in mast cell assays [33, 34] and, using spin-labeled compound 48/80, only the larger polymers bound to mast cells [35]. Increasing the degree of polymerization of protamine sulfate by peptide bond formation with carbodiimide produced increased binding to JB-1 ascites tumor cells and increased growth inhibition. The monomer was inactive, the dimer was more active, and the tetramer was maximally active [36]. The effect of polylysine and polyornithine on mitogen stimulated lymphocyte DNA-synthetic responses has indicated that at low concentrations these compounds stimulated the response while at higher concentrations the response was inhibited [37, 38]. Both these effects may be related to the increased aggregation [39, 40] and capping [41] observed in the presence of polycations. We have also observed stimulation of the response to Con A at low concentrations of Fraction IV while higher concentrations are inhibitory. Maximal stimulation was observed at about 0.6 μg/ml, while the 50% inhibitory dose was about 3–4 μg/ml.

The mechanism by which these polycations exert their effects on cells is not well understood, but it may be mediated by their binding to cellular membranes [31, 36, 42]. Polyamines could also alter the activity of, or serve as substrates for, transglutaminase [43]. Another polyamine derivative, dansyl cadaverine, is a potent inhibitor of this essential enzyme [44]. Obviously, much further work will have to be performed to define the relationship between the structure and polyvalency of the various polycations and their biological effects. Polycationic nature may not be the only requirement for these compounds to be biologically active. It has been shown recently that both compound 48/80 and somatostatin, although differing greatly in cationic nature, have similar three-dimensional structure and are effective degranulators of mast cells [45].

Whatever the mechanism, these polycations have proven to be extremely useful pharmacological tools whose full potential is still being investigated. For example, compound 48/80 has obvious effects on the release of mast cell mediators [46] and is an important tool in discovering how the release of mediators is controlled. Polylysine has shown promise both as an antineoplastic agent [30] and as a carrier of drugs into cells [47]. An examination of the possible structure of the spermine-derived polycations indicates that these compounds are considerably different from both compound 48/80 and polylysine. Compound 48/80 [48] consists of essentially polymerized *p*-methoxy-*N*-methylphenethylamine with the amino groups extending from the polymerized methoxy phenyl groups. Polylysine is similar in that the amino groups extend from a polyamino acid backbone. The spermine-derived polycations differ in that the amino groups are part of the polymerized structure. They should prove to be an interesting addition to the group of polycationic compounds.

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