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Inhibition of Viral Interferon Induction in Mammalian Cell Cultures by Azo Dyes and Derivatives Activated with Rat Liver S9 Fraction

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Four azo dyes (Benzopurpurine 4B, trypan blue, Direct Blue 15, and Congo red) and their derivatives (*o*-tolidine, *o*-dianisidine, and benzidine) were studied for their effect on induction of interferon by influenza virus in mammalian, Rhesus monkey kidney (LLC-MK_2), cell monolayers. Whereas Benzopurpurine 4B, Direct Blue 15, and Congo red inhibited viral interferon induction from approximately 35 to 60%, negligible inhibition was noted with trypan blue and other derivative compounds. By comparison, when rat S9 fraction was used for enzymatic activation of azo dyes and derivatives, interferon inhibition was significantly depressed moreover ($P < 0.01$ to <0.0001) by all chemical compounds. The concentration of rat S9 (0.5%) used for metabolic activation of dyes and congeners was critical because concentrations >0.5% of S9 *per se* inhibited the interferon induction process. Uninduced hamster S9 and both Aroclor 1254-induced hamster and rat S9 fractions were all comparable in their ability to activate the chemical compounds tested. That potential mutagenic and carcinogenic chemicals which require metabolic activation can be discriminated on the basis of interferon induction inhibition in eukaryotic cell cultures augurs for the usefulness and credibility of this system.

INTRODUCTION

Azo dyes are commonly used in coloring cosmetics, pharmaceuticals, and foods, as well as textiles and other commercial products. Increased attention has been focused on these compounds, in particular, those dyes based on benzidine and its analogs (*o*-tolidine, *o*-dianisidine) because of their potential carcinogenicity for humans (Boeniger, 1980). Although a good correlation has been demonstrated between mutagenicity and carcinogenicity, i.e., Ames *Salmonella*/microsome system vs animal bioassays, with numerous groups of chemical compounds (McCann *et al.*, 1975), a similar correlation with azo dyes and their derivatives or reduction products remains tenuous. In part, this may be attributed to certain limitations related to *in vitro* enzymatic activation of azo dyes in the Ames assay system. Most azo dyes require metabolic activation for the release of constituent aromatic amines to activated mutagenic forms (Garner and Nutman, 1977; Hartman *et al.*, 1978; Brown *et al.*, 1978; Lynn *et al.*, 1980), and the pathways, both biologic and chemical, are varied and not fully understood. To overcome these circumstances, concerted efforts have been made to modify the Ames assay by incorporating mutagenicity-enhancing factors, i.e., riboflavin, ATP (Sugimura *et al.*, 1977); uninduced hamster liver S9 fraction, flavin mon-

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onucleotide (Prival and Mitchell, 1982); aerobic and anaerobic preincubation (Brown and Dietrick, 1983) for the reduction of parent azo dyes to mutagenic forms that are amenable to detection by the Ames *Salmonella*/microsome system.

Interferons are inducible proteins formed by activation of cellular genes in response to viral and nonviral microorganisms, and other diverse substances (Stewart, 1981). Although antiviral activity is a common attribute, interferons may also regulate or modulate numerous aspects of the immune system, stimulate or inhibit intracellular enzymes, and influence the proliferation or inhibition of both normal and malignant cells (Taylor-Papadimitriou, 1980). Furthermore, the sensitive nature of the interferon induction process proffers a useful assay, based on inhibition of interferon synthesis, for evaluating the insidious potential of chemicals and particulates of public health concern. Using mammalian cell cultures, adverse activity on interferon induction has been demonstrated with known carcinogens (DeMaeyer and DeMaeyer-Guignard, 1967; Hahon *et al.*, 1979; Sonnenfeld *et al.*, 1980; Sonnenfeld *et al.*, 1983a; 1984; Barnes *et al.*, 1981), asbestos fibers (Hahon and Eckert, 1976), mutagens (Hahon and Ong, 1980), metals (Treagen and Furst, 1970; Gainer, 1972; Hahon *et al.*, 1980; Sonnenfeld *et al.*, 1983b), diesel engine emission particulates (Hahon *et al.*, 1982), and coal fly ash (Hahon *et al.*, 1983). In view of the variety of technical modifications needed to demonstrate the mutagenicity of azo dyes by the Ames *Salmonella*/microsome test, an attempt was made to activate selected azo compounds and congeners with S9 fraction and, subsequently, to note any adverse effects on viral interferon induction. Of further interest in this regard is the recent finding that lymph from gut-associated lymphoid tissue of healthy rabbits possesses antiviral activity indicative of interferon (Bocci *et al.*, 1984). Future investigation of the possible effect of ingested food or products containing azo compounds on interferon levels in abdominal lymph increases the relevance of this study.

This report describes increased inhibition of viral interferon induction in mammalian cell monolayers by four azo dyes (Congo red, trypan blue, Benzopurpurine 4B, and Direct Blue 15) and by their derivatives (benzidine, *o*-tolidine, and *o*-dianisidine), after enzymatic activation by rat liver S9 fraction.

MATERIALS AND METHODS

Viruses and cell cultures. Virus strains and cell lines used in this study were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. The Ao/PR/8/34 influenza and parainfluenza (Sendai) viruses, used for interferon induction and assay, respectively, were prepared from embryonated chicken eggs and assayed for virus infectivity by the immunofluorescent cell-counting technique (Hahon *et al.*, 1973). Rhesus monkey kidney (LLC-MK_2) and human Chang conjunctival (clone 1-5c-4) cell lines obtained from ATCC were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic tissue culture flasks (75 cm^2) with Eagle's minimum essential medium fortified with 100× Essential Vitamin Mixture (10 ml/liter), 200 μM solution L-glutamine (10 ml/liter) to which was added sodium bicarbonate (2.2

g/liter), and 10% fetal bovine serum. Cells were maintained with the aforementioned medium containing 0.5% fetal bovine serum.

Reagents. Trypan blue (Direct Blue 14, *Colour Index* (C.I.) 23,850), and Congo red (Direct Red 28, C.I. 22,120) were purchased from Harleco, Philadelphia, Pennsylvania; Direct Blue 15 (Benzo Sky Blue, C.I. 24,400), *o*-dianisidine, and benzidine were gifts of National Center for Toxicological Research, Jefferson, Arkansas; Benzopurpurine 4B (Direct Red 2, C.I. 23,500), 2-aminoanthracene, and *o*-tolidine were purchased from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of these chemicals were prepared by dissolving specified amounts in 5 ml dimethyl sulfoxide (DMSO) and further diluting in phosphate-buffered saline (PBS), pH 7.1, to the desired concentration. To ensure sterility, solutions were passed through Millex filters GS-0.45 μm (Millipore Corp., Bedford, Mass.).

Liver homogenate 9000*g* supernatant fraction (S9) was prepared from the livers of male Wistar/Lewis rats (225 g/rat) after induction with Aroclor 1254 (ip, 100 mg/kg body wt) as described by Ames *et al.* (1975). For use in experimental tests, 0.5% suspension of S9 homogenate containing 9.3 mg protein/ml (Lowry *et al.*, 1951) was prepared in maintenance medium and then passed through a Nalge Filter Unit 0.45 μm (Nalge Co., Rochester, N.Y.) to obtain sterile preparations. Syrian golden hamster liver S9 fraction, uninduced and Aroclor-induced, were gifts of Dr. W. Z. Whong, of the National Institute of Occupational Safety and Health.

Interferon induction. Duplicate experiments were performed, and the procedure used to study the effect of azo dyes and derivatives on interferon induction was carried out as follows: different concentrations of each dye or chemical in either 10 ml of maintenance medium or 0.5% S9 suspension in maintenance medium was added to 75-cm^2 plastic flasks containing complete LLC-MK_2 cell monolayers (2×10^7 cells) which were then incubated at 35°C for 24 hr. Residual medium was decanted and 2 ml of influenza virus, which had been inactivated by ultraviolet irradiation for 45 sec at a distance of 76.2 mm and wavelength of 253.7 nm, was added onto cell monolayers that were then incubated at 35°C for 2 hr. The multiplicity of infection (m.o.i.) was approximately 2.0. Inoculum was removed and 10 ml of maintenance medium was added to each flask. After incubation at 35°C for 24 hr, supernatant fluid was decanted and centrifuged at 100,000*g* for 1 hr and dialyzed against HCl–KCl buffer, pH 2.0, at 4°C for 24 hr. Dialysis was continued against two changes of PBS, pH 7.1, at 4°C for 24 hr. Fluids were passed through Millex filters GV 0.22 μm to obtain sterile preparations. Samples were stored at −80°C until they were assayed for interferon activity. Preparations with antiviral activity possessed the biological and physical properties ascribed to viral interferons (Lockart, 1973). Controls consisting of cell monolayers which were not treated with test chemical solutions were handled exactly as described above.

Interferon assay. An immunofluorescence cell-counting assay of interferon that had been described previously (Hahon *et al.*, 1975) was used to determine the interferon potency of test samples. Interferon-treated cell monolayers were challenged with 10^4 cell-infecting units of Sendai virus, and infected cells were visualized by direct fluorescent antibody staining. The reciprocal of the interferon

dilution that reduced the number of infected cells to 50% of the control served as the measure of interferon activity, i.e., 50% infected cell-depressing dilution ($ICDD_{50}$). With this assay system, 0.89 interferon unit corresponded to 1.0 unit of National Institutes of Health reference standard Hu IFNB (G-023-902-527).

Statistics. Analysis of variance was used to determine the statistics of significance levels.

RESULTS

Preliminary considerations. Because cell viability is a prerequisite for interferon production, preliminary tests were made to determine the maximum quantity of azo dyes and their respective derivative compounds (Fig. 1) that cells could tolerate without undue loss of viability. Nondividing LLC-MK_2 cells (2×10^7) in monolayers were incubated at 35°C for 24 hr with varied amounts of test chemicals prepared in maintenance medium with and without 0.5% S9. Cell viability was determined by the trypan blue dye exclusion procedure. Cell survival (>95%) was attained with these maximal quantities of chemicals (μmol); Direct Blue 15 (5.04), Congo red (1.43), trypan blue (0.52), Benzopurpurine 4B (1.38), benzidine (0.27), *o*-dianisidine (0.10), and *o*-tolidine (0.47) irrespective of the medium used. Cells tolerated from three- to fiftyfold higher amounts of azo dyes than of the respective derivatives with the exception of trypan blue and its derivative *o*-tolidine which were almost comparable in this respect.

To determine the effect that rat S9 fraction *per se* may have on the viral interferon induction process, cell monolayers were treated with different concentrations of S9 for 24 hr prior to the introduction of virus inducer. Results (Table 1) show that S9 concentrations >0.5% greater increasingly inhibited the production of interferon. Therefore, 0.5% S9 fraction which showed no adverse effect on the interferon induction process was used in subsequent chemical activation experiments.

The carcinogen, 2-aminoanthracene, which requires metabolic activation to

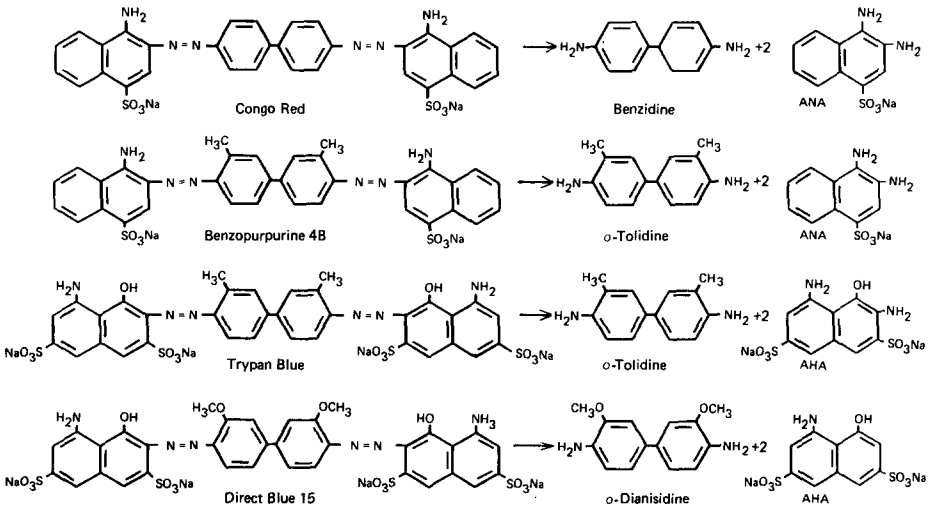

FIG. 1. Structures of azo dyes and their derivatives; ANA, 2-aminonaphthionic acid Na salt; AHA, 2-amino-H acid Na salt.

TABLE 1
EFFECT OF RAT S9 FRACTION CONCENTRATION ON INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS

S9 concentration (%)	Interferon yield[a] ($ICDD_{50}$/ml)	Interferon inhibition[b] (%)
10	26	77.2
5	49	57.1
1.0	79	30.8
0.5	119	0.0
0.1	114	0.0
0 (control)	114	0.0

[a] $ICDD_{50}$, 50% infected cell-depressing dilution.
[b] [(Reciprocal of $ICDD_{50}$/ml of interferon)/(maintenance medium control ($ICDD_{50}$/ml of interferon))] − 1.0 × 100.

potentiate its adverse biological activities, was initially tested with and without S9 fraction to study its effect on viral interferon induction. Results (Table 2) show no significant effect of 2-aminoanthracene on the induction process, however, when the carcinogen was activated by S9, its inhibitory activity on interferon production increased fivefold with the maximal amount of carcinogen tested. When S9 fraction was heated at 80°C for 5 min, the inhibitory effect of 2-aminoanthracene on interferon induction was eliminated. This indicates that the enzymes involved in the activation process became impuissant. Additional tests showed that the highest final concentration (1%) of DMSO used to dissolve dyes and chemicals had no adverse effect on interferon production.

Effect of azo dyes and derivatives on interferon induction. Four azo dyes (Congo red, Direct Blue 15, trypan blue, Benzopurpurine 4B) and their derivatives (benzidine, *o*-tolidine, and *o*-dianisidine) at four different micromole concentrations each, were examined for their effect on viral interferon induction with and without S9 fraction incorporation. Results (Fig. 2) show that the inhibitory activity of Congo red in the presence of S9 on interferon induction was more than doubled with all quantities of dye used as compared to that of Congo red alone. Whereas the derivative of this dye, benzidine, minimally affected interferon production, inhibition of interferon by benzidine with S9 was significantly greater ($P < 0.0001$).

Although Benzopurpurine 4B and Direct Blue 15 in the presence of S9 exhibited significant inhibition of interferon production ($P < 0.01$ and $P < 0.0001$), respectively, as compared to the dyes alone, both azo dyes without preliminary activation, however, were still capable of markedly depressing the interferon process by approximately 50% (Figs. 3,4). The derivatives of these dyes, *o*-tolidine and *o*-dianisidine, had no adverse effect on interferon production, but in the presence of S9 their inhibitory activity on the induction process was >50% at the highest concentrations used. Both trypan blue and its derivative, *o*-tolidine, required activation by S9 before significant inhibition of interferon synthesis occurred (>70% and >50%, respectively; Fig. 5). Although greater inhibitory activity was generally manifested by azo dyes as compared to their derivatives, the quantity

TABLE 2
EFFECT OF 2-AMINOANTHRACENE (2-AA) WITH AND WITHOUT RAT S9 FRACTION ON INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS

2-AA (μmol)	Percentage interferon inhibition[a]	
	S9[b]	Without S9
0.51	57.6	10.6
0.25	45.2	1.5
0.12	12.1	3.0
0.05	0.0	1.4
0 (control)[c]	0.0	0.0

[a] 2-AA, [(Reciprocal of $ICDD_{50}$ of interferon)/(Control (1920 ± $ICDD_{50}$ of interferon))] − 1.0 × 100.
[c] Controls consisted of maintenance medium with or without S9.
[b] Significance level of overall effect of S9, $P < 0.0001$.

of the latter compounds that cells could tolerate without loss of viability, was often several-fold less than that of azo dyes. This was a limiting factor that precluded a valid comparison among the chemical compounds in relation to concentration vs inhibitory activity of viral interferon induction.

Rat versus hamster S9 fraction. Hamster liver S9 homogenate was reported to be superior to rat S9 fraction for the metabolic activation of Congo red and its derivative benzidine to detect mutagens (Prival and Mitchell, 1982). An experiment was performed to compare the efficiency of Aroclor 1254-induced hamster and rat S9 as well as uninduced hamster S9 fraction to activate Congo red and benzidine in relation to inhibition of interferon synthesis. Results (Table 3) show that all hamster and rat S9 fractions were comparable in their ability to activate Congo red or benzidine as evidenced by depressed interferon production. Without activation by S9, the percentage inhibition of interferon production by Congo red was 35% and approximately 80% with S9; inhibition by benzidine was negligible and approximately 50% with S9. With respect to mutagenic activity versus inhi-

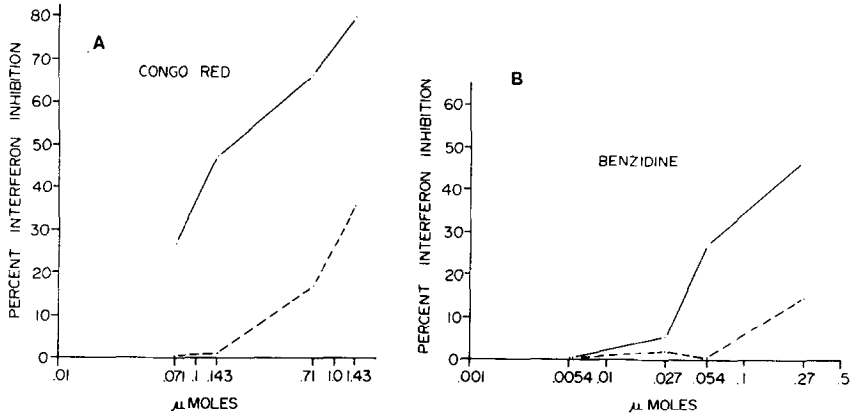

FIG. 2. Inhibition of viral interferon production by Congo red and derivative, benzidine, with (—) and without (- - -) rat S9 fraction.

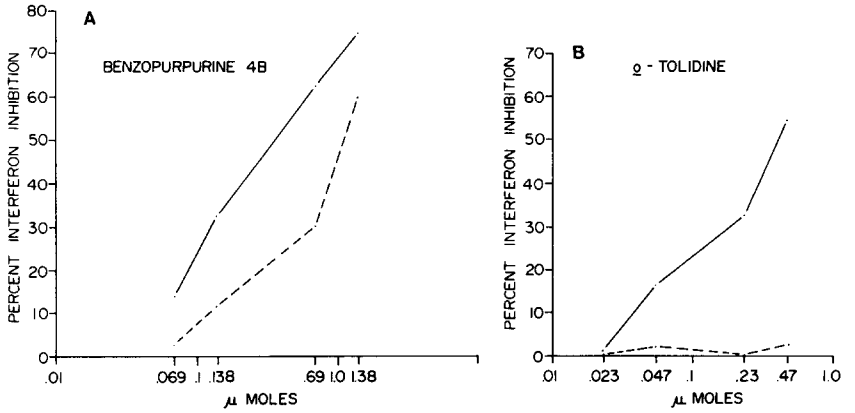

FIG. 3. Inhibition of viral interferon production by Benzopurpurine 4B and derivative, *o*-tolidine, with (—) and without (- - -) rat S9 fraction.

bition of viral interferon induction, manifestation of the former appears to be more discriminatory in the type of S9 fraction required to activate the designated chemical compounds to elicit this entity.

DISCUSSION

The salient finding of this study is that four azo dyes (Benzopurpurine 4B, trypan blue, Direct Blue 15, and Congo red) derived from the mutagenic aromatic amines (*o*-tolidine, *o*-dianisidine, and benzidine), all of which require enzymatic activation to effect mutagenicity, can be activated by rat liver S9 fraction to increase their inhibition of viral interferon induction. Whereas the inhibitory activity varied from negligible to 50% in the absence of S9 fraction, interferon production was significantly depressed moreover when cell cultures were pretreated with S9 fraction in conjunction with maximal noncytocidal quantities of test chemicals.

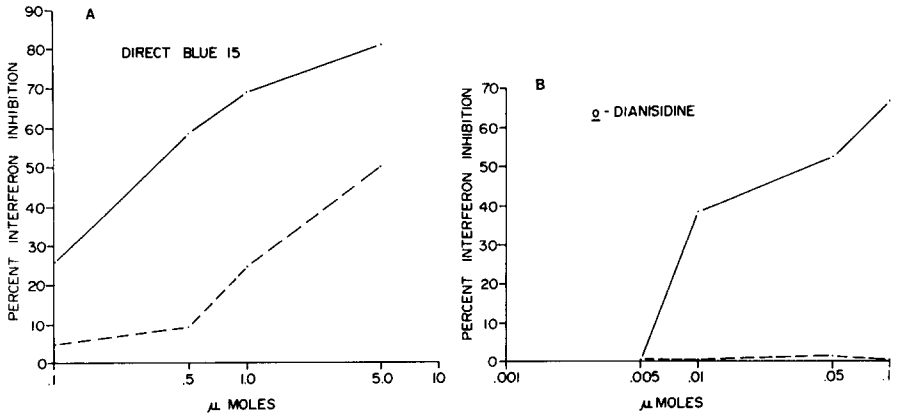

FIG. 4. Inhibition of viral interferon production by Direct Blue 15 and derivative, *o*-dianisidine, with (—) and without (- - -) rat S9 fraction.

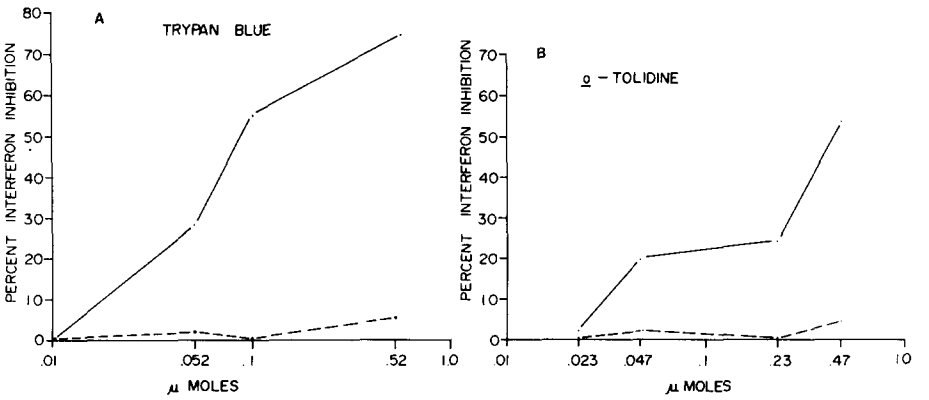

FIG. 5. Inhibition of viral interferon production by trypan blue and derivative, *o*-tolidine, with (—) and without (- - -) rat S9 fraction.

The concentration of S9 fraction used for metabolic activation of dyes and derivatives was critical because concentrations of S9 fraction >0.5% *per se* inhibited the viral interferon induction process. The strong toxicity on mammalian cells by S9 fractions has been a troublesome factor noted in studies involving metabolic activation for assessment of chemical mutagenicity by sister chromatid exchange analysis (White and Hesketh, 1980; Madle, 1981). Throughout this study, 0.5% (9.3 mg protein/ml) of rat S9 was optimal for activating the described chemical compounds without compromising cell viability. It should be noted in this study that, in contrast to various mutagenicity-enhancing factors incorporated into the Ames test with S9 to obtain optimal mutagenic activity of azo dyes and related congeners (Sugimura, 1977; Prival and Mitchell, 1982; Brown and Dietrick, 1983), S9 fraction alone was capable when mixed with either the test azo dyes or derivatives to increase their inhibition of interferon production. This circumstance may be a reflection of the metabolic capabilities or limitations of the different cell types, prokaryotic versus eukaryotic, used in the Ames and interferon inhibition assay systems, respectively. That a system exists in mouse

TABLE 3

EFFECT OF CONGO RED AND DERIVATIVE BENZIDINE ACTIVATED BY RAT OR HAMSTER LIVER S9 FRACTIONS ON THE INHIBITION OF INTERFERON INDUCTION BY INFLUENZA VIRUS IN LLC-MK_2 CELL MONOLAYERS

S9 fraction for activation	Interferon inhibition (%)[a]	
	Congo red[b]	Benzidine
Induced 0.5% rat S9[c]	84.5	54.5
Induced 0.5% hamster S9[c]	78.0	52.8
Uninduced 0.5% hamster S9	79.2	47.6
Maintenance medium (control)	35.3	0.0

[a] [(Reciprocal of $ICDD_{50}$/ml of interferon)/(Overall corresponding control of S9 suspensions ($ICDD_{50}$/ml of interferon) without test chemical)] − 1.0 × 100. Overall control for Congo red (340 $ICDD_{50}$/ml); benzidine (286 $ICDD_{50}$/ml).

[b] Quantities tested, 0.7 μmol Congo red; 0.27 μmol benzidine.

[c] Aroclor 1254-induced S9 liver homogenate.

fibroblasts which can activate some carcinogens in the interferon inhibition system has been suggested from indirect evidence. Reduced glutathione, which can trap active carcinogenic products (Chasseaud, 1979), in conjunction with benzo(*a*)pyrene resulted in negation of the adverse effects of this carcinogen on interferon induction (Sonnenfeld *et al.*, 1980).

Although cell cultures and the interferon induction method differed, our findings agreed with those of Sonnenfeld *et al.* (1984) that benzidine had no detrimental effect on interferon induction. However, we also demonstrated, that with enzymatic activation (rat S9), this carcinogen can markedly inhibit viral interferon induction. When tested by the Ames *Salmonella*/microsome assay, uninduced hamster S9 fraction was superior to rat S9 fraction for activating Congo red and benzidine resulting in strong mutagenic activity (Prival and Mitchell, 1982). In contrast, uninduced hamster S9, and both Aroclor 1254-induced hamster and rat S9 fractions were all capable of activating Congo red and its congener as evidenced by the comparable magnitude of interferon depression. The existence in rat S9 fraction of an inhibitor to the metabolic activation of Congo red and benzidine may account for their weak mutagenic response in the Ames test (Prival and Mitchell, 1982). This factor in rat S9 fraction did not impede activation of the aforesaid chemicals and their subsequent detrimental effect on the cellular defense mechanism under investigation. As noted herein, Aroclor 1254-induced and uninduced hamster S9 were comparable in their ability to activate both Congo red and its derivative, benzidine. Uninduced hamster S9, apparently, contains sufficient levels of hepatic microsomal enzymes that are effective in activating these azo compounds to metabolites capable of reducing interferon production without resorting to drug-induction to enhance levels and activities of hepatic S9 cytochrome *P*-450 enzymes.

That the activation by microsomal enzymes of the azo dye derivatives, *o*-tolidine, *o*-dianisidine, and benzidine, tested herein, resulting in their ability to inhibit interferon induction, indicates that other reduction products may be involved in the cited phenomenon. Urinary metabolites of benzidine, both *N*-acetylbenzidine (AB), and *N, N'*-diacetylbenzidine (DAB) were reported to show stronger mutagenicity than benzidine itself (Tanaka *et al.*, 1981). That benzidine metabolism could include the following sequence: benzidine → AB → DAB → *N*-hydroxy-*N,N'*-diacetylbenzidine → nucleic acid binding with the latter compound exhibiting mutagenic activity has been reported by Morton *et al.* (1979). The metabolic pathways followed by azo dyes are complex and dependent on numerous interrelated factors (Walker, 1970). Of the azo compounds studied herein, the specific metabolites or reduction products responsible for the inhibition of interferon induction are undetermined. Nonetheless, with the demonstration that potential mutagenic and carcinogenic chemicals which require metabolic activation, can be discriminated on the basis of interferon induction inhibition in mammalian cell cultures, augurs for the usefulness and credibility of this system.

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