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EFFECT OF INTERFERON SYNTHESIS UPON THE METABOLISM OF [CARBOXYL-^{14}C]-ASPIRIN IN THE MOUSE

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Abstract—The effect of stimulation of interferon (IFN) synthesis with the immunomodulators, polyriboinosinic polyribocytidylic acid (poly rI:rC) and Newcastle Disease Virus (NDV) upon the *in vivo* metabolism of [*carboxyl*-^{14}C]-aspirin was investigated in male mice of three different strains. Following poly rI:rC administration to DBA/2 mice, the metabolic conjugation of salicylic acid with glycine was significantly increased, glucuronidation was little changed and there was an approximately 10-fold reduction in the excretion of the oxidation product, gentisic acid. Prolongation of hexobarbitone-induced sleeping time confirmed the ability of this agent to depress oxidative metabolism *in vivo*. Poly rI:rC administration to C57BL/6By and BALB/cBy mice resulted in similar changes in aspirin metabolism *in vivo*. Treatment of C57BL/6By mice with NDV produced high levels of circulating IFN and produced alterations in aspirin metabolism similar to those seen after poly rI:rC treatment. Comparable studies in BALB/cBy mice, which did not produce detectable levels of IFN in response to NDV challenge, revealed no change in the fate of aspirin metabolism following treatment with this agent. These data indicate that the *in vivo* oxidation of salicylate, but not its conjugation, can be depressed, at least indirectly, by an interferon-associated mechanism.

The activity of the immune system may be influenced by a wide variety of chemical and physical agents collectively known as immunomodulators. Many such agents, after *in vivo* administration, have also been shown to depress the activity of the cytochrome P-450-dependent mixed-function oxidase system, apparently involving depressed synthesis of cytochrome P-450 apoprotein resulting in a reversible reduction in hepatic levels of the active holoenzyme [1]. As a consequence, the *in vitro* activities of a number of associated drug oxidations are subsequently depressed [1]. This impairment of oxidative drug metabolism seems to be, at least indirectly, associated with the induction of interferon (IFN) synthesis since, (a) many agents, seemingly only related by their common ability to induce IFN, are also capable of depressing drug oxidation [2], (b) the two processes are apparently temporally related [3], and (c) *in vivo* administration of purified IFN reduces cytochrome P-450 levels and associated oxidative activities *in vitro* [4, 5].

The metabolism of drugs and other xenobiotics in the animal body is typically a biphasic process, in which the compound first undergoes a Phase I reaction of oxidation, reduction or hydrolysis. This serves to introduce or reveal within the foreign compound a functional group which serves as the site for a Phase II reaction, or conjugation, in which the compound is linked with one of a range of endogenous moieties. The majority of work reported so far has been concerned with the influence of altered immune status upon the oxidative Phase I reactions of drug metabolism. However, the clearance of the great majority of xenobiotics from the body depends upon their conjugation, and it is therefore of interest to investigate possible effects of immunomodulators upon drug conjugation.

The widely used minor analgesic, aspirin (acetylsalicylic acid, ASA), undergoes facile hydrolysis in the body releasing salicylic acid (SA) which is excreted in the urine of animals or man almost exclusively conjugated either with glycine as salicyluric acid (SUA) or with glucuronic acid, giving rise to both ester (minor) and phenolic (major) salicylglucuronides (SG). Oxidation, yielding gentisic acid (GA, 2,5-dihydroxybenzoic acid), is a minor route of metabolism, and a small proportion of the dose is eliminated as free SA [6]. ASA is thus an appropriate probe compound to use to study the influence of immunomodulation, specifically IFN induction, upon Phase II drug metabolism.

We have investigated the influence of two different immunomodulators and inducers of IFN, polyriboinosinic polyribocytidylic acid (poly rI:rC) and Newcastle Disease Virus (NDV), upon the *in vivo* metabolism of ASA. While poly rI:rC is a potent IFN inducer [7] and depresses drug oxidation [2] many other biological processes may be affected by this synthetic polynucleotide [8]. Consequently, in order to investigate more directly the influence of IFN induction upon the *in vivo* metabolism of ASA a more specific model was needed. To this end we have used two strains of mice, BALB/cBy and C57BL/6By, whose IFN responses to NDV are determined by a single, co-dominant, autosomal gene, designated IF-1 [9]. "Low" strains such as BALB/cBy, homozygous for the low (l) allele at this locus and of genotype IF-1^{l}, produce low, often

undetectable, serum levels of IFN after NDV challenge. In contrast, "high" strains, such as C57BL/6By, which are homozygous for the high (h) allele, and are of genotype IF-1^h, will respond to NDV by producing high, readily measured serum levels of IFN. In contrast poly rI:rC induces the synthesis of IFN by mechanism(s) not involving the IF-1 locus and is an effective IFN inducer in all strains of mice [10], thus permitting its use as a positive control.

In this paper we report the results of investigations into: (1) The influence of poly rI:rC upon the *in vivo* metabolism of [*carboxyl*-^{14}C]-ASA and upon the activity of the P-450 mixed-function oxidase system, as determined by hexobarbitone-induced sleeping time (HST), in DBA/2 mice, and (2) the influence of poly rI:rC and NDV on the *in vivo* metabolism of [*carboxyl*-^{14}C]-ASA in BALB/cBy and C57BL/6By mice.

MATERIALS AND METHODS

Compounds

[*carboxyl*-^{14}C]-Aspirin, sp. activity 34.3 mCi/mmol, radiochemical purity >98% was purchased from New England Nuclear Research Products, Stevenage, U.K. Salicyluric (*o*-hydroxyhippuric) acid, gentisic acid, salicylic acid, aspirin, poly rI:rC (Na salt) and β-glucuronidase ("Glucurase"), were all purchased from Sigma Chemical Co, Poole, Dorset, U.K. Hexobarbitone was obtained from May & Baker Ltd, Dagenham, U.K. A live attenuated strain of NDV, Hitchner B1, was obtained as a freeze dried vaccine ("Newcadin") from Glaxo Animal Health Ltd, Uxbridge, U.K.

Animals

Male DBA/2, C57BL/6By and BALB/cBy mice, 8–12 weeks old, 20–30 g b.w., were from colonies maintained by the Animal Department of St Mary's Hospital Medical School. Animals were fed *ad libitum* on Labsure CRM pellets (K and K-Greef Ltd, Croydon, U.K.). The original breeding stocks of the C57BL/6By and BALB/cBy mice were the kind gift of Dr B. Loveland, CRC, Harrow, U.K.

Thin layer chromatography

Thin layer chromatography (TLC) was performed essentially according to Hutt *et al.* [6] using silica gel F_{254} (Cat. No. 5554, E. Merck A.G., Darmstadt, F.R.G.) 0.2 mm thick on aluminium support. Aliquots (20 μl), of 0–24 hr urine samples were applied directly to the TLC plates, which were developed with chloroform : ether : acetic acid : methanol (120 : 60 : 18 : 1 by vol). Metabolites were identified by comparing R_f values with those of authentic standards and by colour reaction with the following sprays, (1) p-dimethylaminobenzaldehyde (1 g) in acetic anhydride (25 ml) and sodium acetate (100 mg); glycine conjugates appear as orange/red spots after heating at 100° for 5 min. (2) 5% $FeCl_3$ in 0.5 M HCl; SA and GA appear as blue spots. (3) 5 : 1 mixture of 0.2% naphtharesorcinol in ethanol and 85% orthophosphoric acid; glucuronic acid derivatives yield a blue spot on a pink background on heating at 100° for 10 min. R_f values of reference standards were: SUA 0.3, GA 0.66 and SA 0.84.

Urine treatment

Samples of 0–24 hr urine were incubated with β-glucuronidase (24 hr/37°) and the incubation mixture run directly on the TLC system.

Radiochemical techniques

The ^{14}C content of solutions and excreta were determined by liquid scintillation spectrometry using Cocktail T scintillation fluid (BDH Chemicals Ltd, Poole, U.K.) with a Packard TriCarb instrument, Model 4640 (Packard Instrument Co, Caversham, Reading, U.K.). ^{14}C on chromatograms was quantified by cutting the TLC plates into 1 cm horizontal strips and without further extraction counting these for ^{14}C. Counting efficiency was assessed by reference to an external standard, using a regularly determined quench correction curve.

Drug treatments

(A) *Poly rI:rC.* Poly rI:rC was prepared as a 1 mg/ml solution in sterile, pyrogen-free saline and allowed to dissolve at 4° overnight with gentle mixing immediately prior to use. DBA/2, BALB/cBy or C57BL/6By mice received poly rI:rC, i.p. (10 mg/kg bw), or an equivalent volume of dose vehicle as control.

(B) *NDV.* The contents of one vial of "Newcadin" vaccine, equivalent to 10^{10} Egg Infective $Dose_{50}$ (EID_{50}), was dissolved in 10 ml of sterile, pyrogen-free saline. BALB/cBy and C57BL/6By, mice received either 0.1 ml (10^8 EID_{50}) of this preparation i.v. via a tail vein or poly rI:rC as described above. Control mice received saline vehicle either i.v. or i.p. as appropriate.

(C) [*carboxyl*-^{14}C]-*aspirin administration.* Twenty-four hours after pretreatment all mice received [*carboxyl*-^{14}C]-ASA orally as a solution in aq. $NaHCO_3$. The dose level for DBA/2 mice was 100 mg/kg b.w. (5 μCi/mouse) while BALB/cBy and C57BL/6By mice received 10, 50 or 100 mg/kg b.w. (5 μCi/mouse). Mice were housed individually in glass metabolism cages ("Mini-Metabowls", Jencons Scientific Ltd, Leighton Buzzard, U.K.), food and water were provided *ad libitum* and urine and faeces collected daily for three days.

Determination of HST

Dose solutions of hexobarbitone were prepared immediately prior to use by dissolving an accurately weighed amount in the stoichiometric equivalent of 1 M NaOH and diluting with water to a concentration of 8.5 mg/ml. This was injected i.p. in a volume of 0.01 ml/g b.w., to give a dose of hexobarbitone of 85 mg/kg b.w. HST was taken as the duration of the loss of the righting reflex.

Determination of serum IFN levels

Serum IFN levels were determined 24 hr after the following treatments: (a) in DBA/2 mice after poly rI:rC treatment (N = 4) and after saline alone (N = 2); (b) in BALB/cBy mice after NDV treatment (N = 4) and after saline (N = 2); and (c) in C57BL/6By mice after NDV treatment (N = 4) and after

Table 1. Percentage recovery of ^{14}C-aspirin in the 0–24 hr urine of DBA/2, BALB/cBy and C57BL/6By mice given [^{14}C]-aspirin following treatment with either saline, poly rI:rC or NDV

Dose (mg/kg)	Strain	Treatments: Saline	Poly rI:rC (% Dose in 0–24 hr urine)	NDV
10	DBA/2	—	—	—
	BALB/cBy	74.0 ± 10.5	73.0 ± 14.3	79.7 ± 14.3
	C57BL/6By	79.9 ± 15.3	77.2 ± 14.9	89.6 ± 5.1
50	DBA/2	—	—	—
	BALB/cBy	70.6 ± 4.3	68.0 ± 6.1	83.7 ± 7.7
	C57BL/6By	60.8 ± 28.2	63.9 ± 39.4	68.8 ± 39.2
100	DBA/2	86.3 ± 6.8	77.4 ± 10.8	—
	BALB/cBy	73.0 ± 6.0	83.6 ± 7.1	78.6 ± 12.8
	C57BL/6By	86.3 ± 5.5	61.9 ± 29.4	89.5 ± 3.1

Mice were treated with either saline (0.1 ml i.v.), poly rI:rC (10 mg/kg i.p.) or NDV (0.1 ml i.v.) as appropriate, 24 hr later all mice received [*carboxyl*-^{14}C]-aspirin orally as a solution in aqueous $NaHCO_3$ at a dose of either 10, 50 or 100 mg/kg b.w. Urine and faeces were collected daily for 3 days and 0–24 hr urinary recovery of the dose determined by liquid scintillation counting.

Values are means ± SD (N = 3–8).

saline (N = 2). Following exsanguination under light ether anaesthesia serum IFN was determined by a viral RNA synthesis assay using murine L-cells challenged with Semliki Forest Virus. The assay was standardised against murine IFN international standard G002-904-511 (National Institute for Allergy and Infectious Diseases, NIH, Bethesda, MD). Assay detection limits were 16 std units/ml.

Statistical analysis

All data were analysed using Student's *t*-test with a level of significance for P of <0.05.

RESULTS

(1) *The influence of poly rI:rC upon HST and* ^{14}C-*aspirin metabolism in DBA/2 mice*

Influence of poly rI:rC upon HST. Poly rI:rC pretreatment resulted in a significant prolongation of HST from 42.1 ± 7.3 min to 70.2 ± 17.2 min (mean ± SD; N = 12; P < 0.01).

Influence of poly rI:rC upon excretion of ^{14}C *after administration of* ^{14}C-*aspirin.* In both poly rI:rC and control groups some 90% of the dose was recovered in 3 days with the bulk in the 0–24 hr urine (Table 1). ^{14}C excretion was not significantly different between poly rI:rC treated or control groups for either the 0–24 hr or the 0–72 hr collection periods.

Influence of poly rI:rC upon the excretion of urinary metabolites of ^{14}C-*aspirin.* Radio-TLC of the 0–24 hr urine revealed four ^{14}C-containing bands. Comparison of R_f values and colour reactions of these bands with those of authentic standards identified them as SUA (R_f 0.30), GA (R_f 0.66), SA (R_f 0.84). The radioactive band, R_f 0.0, was identified as salicylglucuronide by colour reaction and by its disappearance following β-glucuronidase treatment with a corresponding increase in the amount of radioactivity running with salicylic acid. These bands were quantified as described above and the results are shown in Fig. 1. Treatment with poly rI:rC resulted in a small but significant increase in urinary excretion of SUA from 34.4 ± 2.9 to 45.4 ± 7.0 (mean ± SD, P < 0.01) and a highly significant decrease in that of GA from 3.2 ± 0.7 to 0.3 ± 0.2 (mean ± SD, P < 0.001).

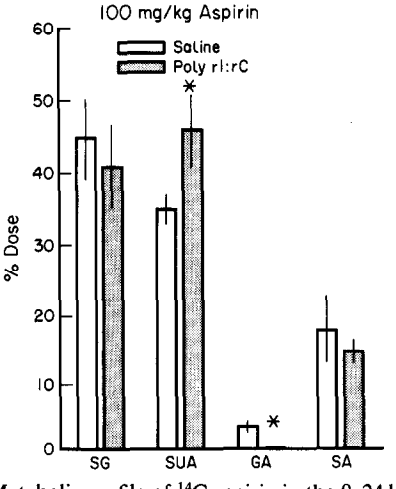

Fig. 1. Metabolic profile of ^{14}C-aspirin in the 0–24 hr urine of DBA/2 mice following treatment with either saline or poly rI:rC. Values are means ± SD (N = 6); * P < 0.05 compared to control; SG, salicylglucuronides; SUA, salicyluric acid; GA, gentisic acid; SA, salicylic acid. Mice were treated with either saline (0.1 ml i.v.) or poly rI:rC (10 mg/kg i.p.) as appropriate. Twenty-four hours later all mice received [*carboxyl*-^{14}C]-aspirin orally as a solution in aqueous $NaHCO_3$ at a dose of 100 mg/kg b.w. Urine and faeces were collected daily for 3 days and 0–24 hr urinary metabolites assayed by radio-TLC.

Serum IFN levels in DBA/2 mice after poly rI:rC treatment. Serum IFN levels 24 hr after treatment in four mice were 208, 104, 259 and 127 std IFN units/ml while levels in control mice were below the level of detection.

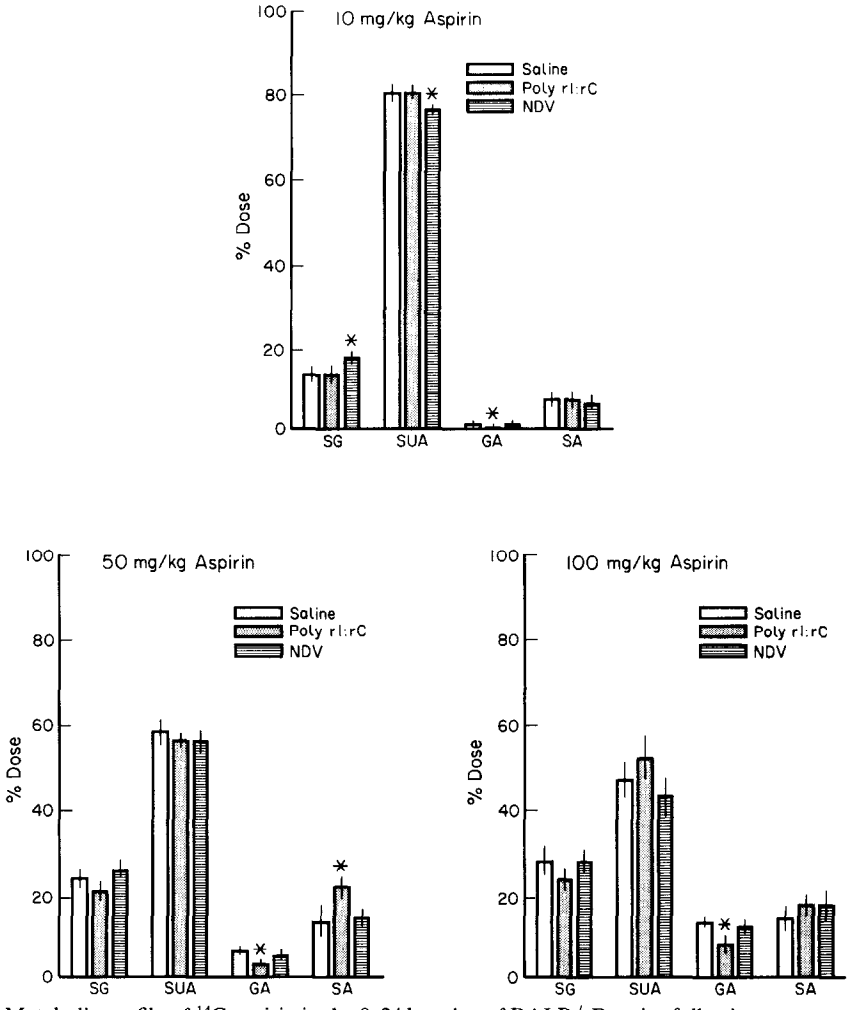

Fig. 2. Metabolic profile of ^{14}C-aspirin in the 0–24 hr urine of BALB/cBy mice following treatment with either saline, poly rI:rC or NDV. Values are means ± SD (N = 6); * $P < 0.05$ compared to control; SG, salicylglucuronides; SUA, salicyluric acid; GA, gentisic acid; SA, salicylic acid. Mice were treated with either saline (0.1 ml i.v.), poly rI:rC (10 mg/kg i.p.) or NDV (0.1 ml i.v.) as appropriate. Twenty-four hours later all mice received [*carboxyl*-^{14}C]-aspirin orally as a solution in aqueous $NaHCO_3$ at a dose of either 10, 50 or 100 mg/kg b.w. Urine and faeces were collected daily for 3 days and 0–24 hr urinary metabolites assayed by radio-TLC.

(2) *The effect of NDV and poly rI:rC upon* ^{14}C-*aspirin metabolism in BALB/cBy and C57BL/6By mice*

Influence of poly rI:rC and NDV upon excretion of ^{14}C *after administration of* ^{14}C-*aspirin.* ^{14}C was extensively eliminated in the urine of mice from all groups with 60%–85% being recovered during the first 24 hr (Table 1). Application of Student's *t*-test to these data showed there were no significant differences in the proportions of ^{14}C excreted in the first 24 hr between control, poly rI:rC or NDV treated mice at any dose.

Influence of poly rI:rC and NDV upon excretion of urinary metabolites of ^{14}C-*aspirin.* The urinary metabolites SG, SUA, GA and SA were identified in all mice and quantified by the methods described above. The proportion of the dose excreted as SUA by both strains in the first 24 hr decreased with the increase in ASA does while the proportions of SG, GA and SA all increased with increasing dose (Figs 2 and 3). This effect was more pronounced in C57BL/6By mice. These trends were independent of the treatment regime.

The effect of NDV or poly rI:rC treatment upon the 0–24 hr urinary excretion of these metabolites in BALB/cBy and C57BL/6By mice is presented in Figs 2 and 3 respectively. Treatment with NDV or poly rI:rC had little effect upon the pattern of salicylate conjugation in either BALB/cBy or C57BL/6By mice. However, following poly rI:rC treatment, in C57BL/6By mice there were small increases in the proportion of SUA excreted in the first 24 hr which were significant ($P < 0.05$) at all dose levels except 50 mg/kg. This effect was not observed in BALB/cBy mice. Neither poly rI:rC nor NDV treatment significantly altered the excretion of SG by either strain.

The excretion of the oxidation product, GA, was

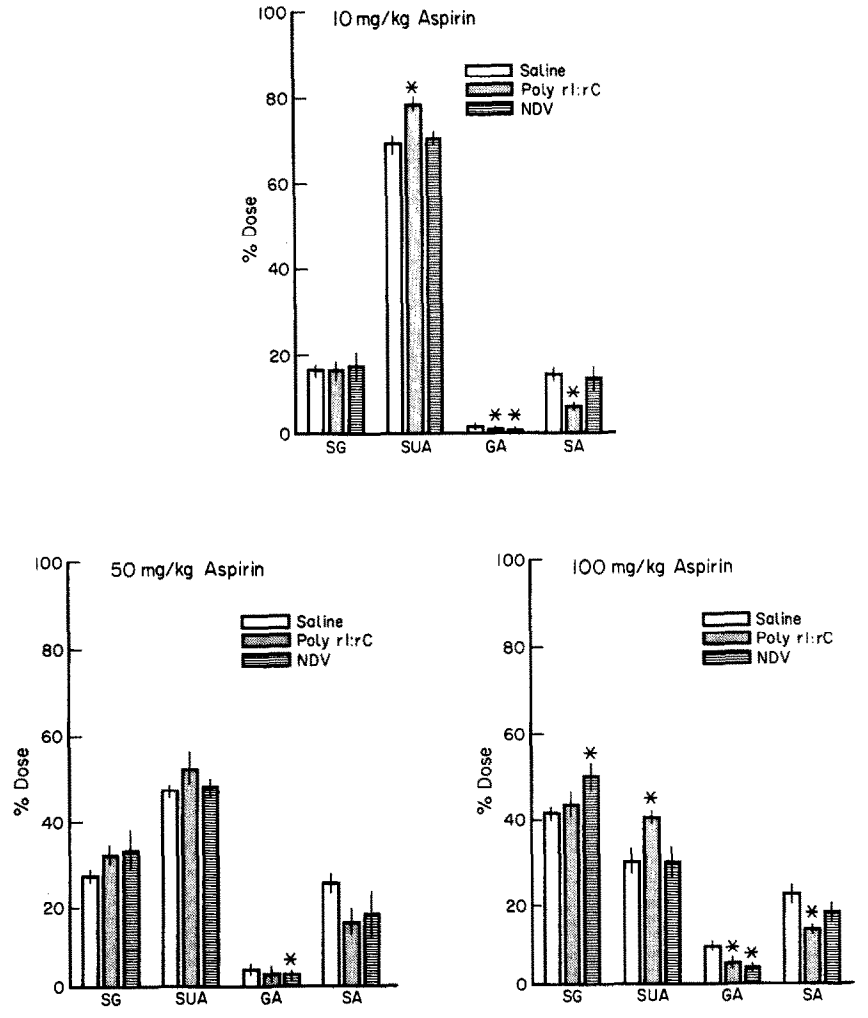

Fig. 3. Metabolic profile of ^{14}C-aspirin in the 0–24 hr urine of C57BL/6By mice following treatment with either saline, poly rI:rC or NDV. Values are means ± SD (N = 6); * $P < 0.05$ compared to control; SG, salicylglucuronides; SUA, salicyluric acid; GA, gentisic acid; SA, salicylic acid. Mice were treated with either saline (0.1 ml i.v.), poly rI:rC (10 mg/kg i.p.) or NDV (0.1 ml i.v.) as appropriate. 24 hr later all mice received [*carboxyl*-^{14}C]-aspirin orally as a solution in aqueous $NaHCO_3$ at a dose of either 10, 50 or 100 mg/kg b.w. Urine and faeces were collected daily for 3 days and 0–24 hr urinary metabolites assayed by radio-TLC.

reduced in both strains and at all dose levels by poly rI:rC treatment. This was significant ($P < 0.05$) in each case except the 50 mg/kg dose to C57BL/6By mice. Following NDV treatment, C57BL/6By mice showed a significant decrease ($P < 0.05$) in the excretion of GA at each dose level. In contrast, excretion of GA by BALB/cBy mice was unaffected by NDV treatment at any dose.

Serum IFN levels in BALB/cBy and C57BL/6By mice after NDV treatment. Twenty-four hours following NDV treatment, serum IFN was found in C57BL/6By mice but not in BALB/cBy mice. The levels in the four C57BL/6By mice tested were 40, 66, 73 and 45 std IFN units/ml.

DISCUSSION

The ability of the immunomodulator poly rI:rC to depress the oxidative metabolism of xenobiotics [2] was confirmed here by the ability of this agent to prolong HST 24 hr after administration to DBA/2 mice. Similar increases in HST were reported by Floersheim *et al.* [11] when poly rI:rC was administered to Swiss mice.

The *in vivo* metabolic conjugation of salicylate in mice appears to be largely unaffected by poly rI:rC pretreatment except for a small but significant increase in the proportion of SUA found in the urine of DBA/2 and C57BL/6By mice. Interestingly, this was not found in BALB/cBy mice. The significance of this observation is presently unclear. In contrast there was an approximately 10-fold reduction in the proportion of the dose excreted as the oxidation product, GA, in the 0–24 hr urine of DBA/2 mice, and a smaller, but still significant decrease in GA excretion in BALB/cBy and C57BL/6By mice after pretreatment with poly rI:rC. This depression of the *in vivo* oxidation of ASA further illustrates the ability of poly rI:rC to depress mixed-function oxidase activity.

In BALB/cBy and C57BL/6By mice the decrease in the proportion of the dose excreted as SUA as the dose increased indicates capacity limited kinetics for this pathway. Kinetics of this type for the conjugation of salicylate with glycine are well known in man [12] and rat [13]. Within each strain this effect was independent of the immunomodulator used and so did not interfere with the interpretation of the data.

NDV treatment had no systematic effect upon the pattern of salicylate conjugation in either BALB/cBy or C57BL/6By mice, although some small statistically significant differences were noted. Gentisic acid formation was significantly reduced at all dose levels in the C57BL/6By strain, from 1.5% to 0.9% of the recovered dose at 10 mg/kg, from 4.0% to 2.6% at 50 mg/kg and from 8.6% to 3.8% at 100 mg/kg. This pathway was apparently unaffected in BALB/cBy mice following NDV treatment.

That circulating levels of IFN could only be measured in C57BL/6By and not BALB/cBy mice following challenge with NDV is in accord with the strain differences originally described by De Maeyer and De Maeyer-Guignard [9]. Depression in salicylate oxidation was observed only in this strain following NDV treatment, while poly rI:rC depressed this pathway in both C57BL/6By and BALB/cBy mice.

Singh and Renton [14] were able to demonstrate that IFN induction results in the depression of hepatic levels of cytochrome P-450 by using the same IFN induction model. They were able to show that there was a correlation between the circulating levels of IFN in C57BL/6By mice 24 hr after NDV challenge, and the extent of loss of hepatic cytochrome P-450 and depression in aminopyrine N-demethylation activity *in vitro*. There was no loss of cytochrome P-450 or the associated N-demethylase activity in C_3H/HeJ mice which are "low" IFN responders to NDV like the BALB/cBy strain used here.

The results reported here further demonstrate that agents able to modulate the activity of the immune system of a host, such as poly rI:rC and NDV, can influence the *in vivo* oxidative metabolism of xenobiotics. They also strongly suggest that this interaction involves mechanisms concerned with the synthesis of IFN. How direct such an interaction may be or through what possible mechanisms it may be mediated is presently unclear [1]. In contrast to this the *in vivo* conjugation of salicylate appeared to be little affected by either poly rI:rC or NDV. However, it will be necessary to investigate other substrates, chosen to exemplify various other conjugation reactions, and to study the effects of other immunomodulators, before firm conclusions can be drawn as to the effects of immunomodulation on the enzyme systems responsible for drug conjugation.

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