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HEPATIC GROWTH EFFECTS OF METHYLPREDNISOLONE, AZATHIOPRINE, MYCOPHENOLIC ACID, AND MIZORIBINE[1]

Cyclosporine (CSA) and FK506 augment liver regeneration after partial hepatectomy in rats (*1, 2*), an effect that is not the consequence of immune modulation (*3*). In addition, both drugs increase the hepatocyte proliferative response in dogs that is characteristic after Eck fistula, and they also prevent the hepatocyte atrophy and organelle disruption that is caused by this operation (*4*). Substances causing these changes have been termed *hepatotrophic*. Rapamycin (RPM),* a third potent immunosuppressant, has opposite (antihepatotrophic) actions without evidence of inherent hepatocyte toxicity (*5*).

A hypothesis that these growth control alterations are caused by altered signal transduction of the hepatocytes independent of immunologic pathways (*4*) has been supported by in vivo and in vitro experimentation (*3*). However, there is a paucity of information about the influence of other immunosuppressive drugs on growth and regeneration. Using in vivo (partial hepatectomy) and in vitro (tissue culture) rat models described elsewhere (*2, 3, 5, 6*), we have investigated the effect upon hepatocyte proliferation of methylprednisolone (MP), mycophenolic acid (MPA), mizoribine (MZ), and azathioprine (AZA).

In vivo experiments were performed with adult male inbred Fisher 344 rats weighing 180–200 g (Hilltop Lab Animals, Scottsdale, PA). The rats were given standard rat laboratory diet and water ad libitum, and kept in a temperature and light-controlled room (light 0730–1930). Immunosuppressive drugs were given preoperatively on days −3, −2, and −1, and as a final dose just after 70% hepatectomy. The operations were performed between 0900 and 1030 under light ether anesthesia (*2, 5*). Immediate resumption of food and drink made it unnecessary to provide parenteral fluid and electrolyte support. At 24 hr after the hepatectomy when the rat liver regeneration response is at its peak, 185×10^4 Bq ^{3}H-thymidine was administered by intraperitoneal injection, followed 2 hr later by guillotine killing. Hepatic DNA was extracted and purified as previously described (*2, 5*). DNA content was measured with a standard of calf thymus DNA (Sigma, St. Louis). Mitotic indices were obtained in 3 animals in the groups 1, 3, 5, 7 and 9, and in 6 animals in the groups 2, 4, 6, 8 and 10 (Table 1) that were sacrificed at 33 hr. The liver specimens from these animals were formalin-fixed, stained with H&E, and examined for the percentage of mitoses.

The in vivo experimental groups are summarized in Table 1. After partial hepatectomy, the contemporaneous vehicle controls showed considerable variability from group to group (Fig. 1). In control experiments, the highest DNA synthesis and mitotic rates were in control animals injected with saline (Table 1, groups 1 and 3) whereas the lowest were in animals with drug vehicle containing CMC solvent alone (group 9) and CMC mixed with Tween and alcohol (group 5). This suggested a potential inhibitory effect of the CMC carrier and emphasized the need for concurrent controls for each test group.

The only drug that significantly augmented regeneration relative to the controls was MP, $P<0.01$ (Fig. 1). The only drug that significantly inhibited regeneration was MZ ($P<0.01$). The MZ inhibition was profound, reducing DNA synthesis >85% and the mitosis rate >70% (Fig. 1). MPA at a standard dose and AZA at low (1 mg/kg oral) and high (6 mg/kg i.m.) doses were without effect on regeneration in the whole animal (Fig. 1).

In vitro experiments were performed with hepatocyte culture preparations made from the livers of the same strain of unal-

TABLE 1. Conditions of in vivo experiments[a]

Group	Drugs	Dose used (mg/kg/dose)	Route	Vehicle
1 (n=8)	—	—	IM	Saline
2 (n=13)	MP	1	IM	Saline
3 (n=8)	—	—	IM	Saline
4 (n=13)	MZ	20	IM	Saline
5 (n=8)	—	—	PO	0.5% CMC; 0.4% Tween 0.9 alcohol in saline
6 (n=13)	MPA	15	PO	0.5% CMC; 0.4% tween 0.9% alcohol in saline
7 (n=8)	—	—	PO	Olive oil
8 (n=13)	AZA	1	PO	Olive oil
9 (n=8)	—	—	IM	1% CMC in saline
10 (n=13)	AZA	6	IM	1% CMC in saline

[a] Methylprednisolone (MP) and mizoribine (MZ) were dissolved in saline; mycophenolic acid (MPA) was dissolved in 0.5% carboxymethylcellulose (CMC), 0.4% Tween and 0.9% alcohol in saline; azathioprine (AZA) was mixed with olive oil or dissolved with 1% CMC in saline. Drug solutions were made daily for each dose of each drug.

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* Abbreviations: AZA, azathioprine; MP, methylprednisolone; MPA, mycophenolic acid; MZ, mizoribine; RPM, rapamycin.

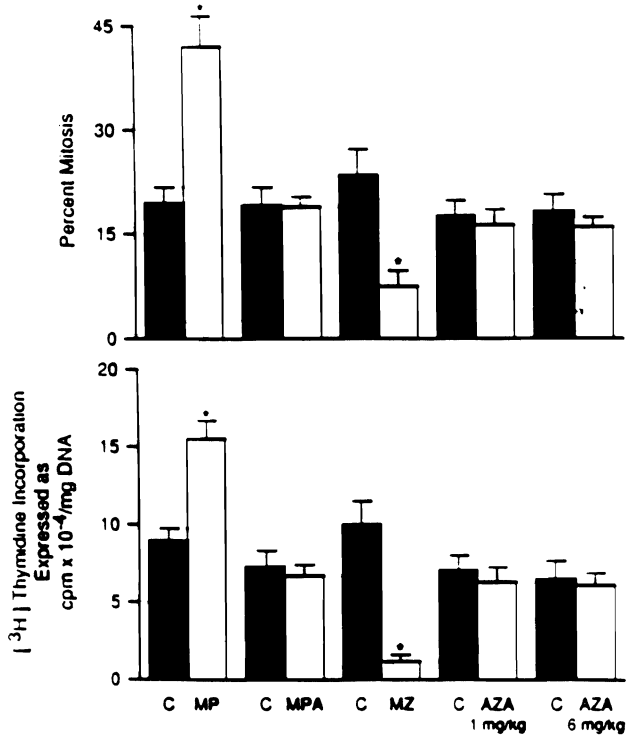

FIGURE 1. ^{3}H-thymidine incorporation and mitoses/1000 hepatocytes in 70% hepatectomized rats treated or not treated with methylprednisolone (MP), mycophenolic acid (MPA), mizoribine (MZ), and azathioprine (AZA). Drug doses are as shown in Table 1. Twenty-one animals were used for each drug: 8 rats were used as control and 13 were treated with the drugs. Three rats of each control group and six of each experimental group were used for determinations of the number of mitoses. Bars are mean ± SD. (*)P<0.01 (drug versus control).

TABLE 2. Viability, DNA concentration, and transaminase levels of hepatocytes in media enriched with immunosuppressive drugs in the absence of EGF[a]

	24 hr		48 hr		
	Viability percentage	DNA (μg/dish)	Viability percentage	DNA (μg/dish)	ALT (U/ml)
Control	95±4	3.2±0.2	96±3	3.3±0.2	5.1±1
MPA 10 μg/ml	94±3	3.3±0.3	96±4	3.3±0.2	4.1±0.6
AZA 1 μg/ml	56±4	2.1±0.3	35±5	1.7±0.3	16±3
MZ 100 ng/ml	95±6	3.1±0.2	95±9	3.3±0.2	4.5±0.7
MP 10 μg/ml	94±3	3.3±0.3	97±3	3.3±0.2	5.1±0.8

[a] MPA: mycophenolic acid; AZA: azathioprine; MZ: mizoribine; MP: methylprednisolone.

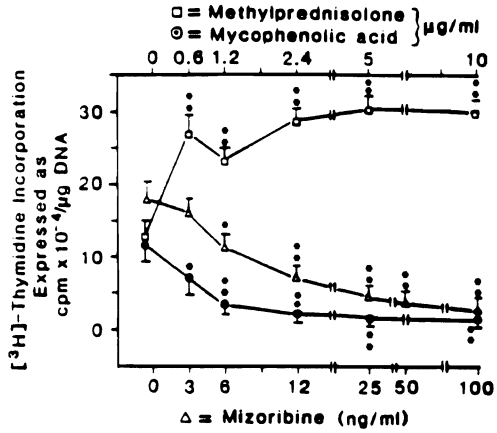

FIGURE 2. Doses versus DNA synthesis response with MP, MPA, and MZ of cultured hepatocytes (n=9 for all data points [3 experiments with triplicate determinations). Mean ± SD; (*)P<0.05; (**)P<0.01.

tered male rats (180 and 200 g). Hepatocytes were isolated with a previously standardized in situ two-step collagenase perfusion technique (*3, 6*). The hepatocytes were dispersed and washed twice with cold CA^{++}-free perfusion buffer and resuspended in basal medium (MEM) supplemented with pyruvate (1 mM), proline (0.26 mM), insulin (10^{-7} M), and 5% FCS. Cell number was determined with a hepatocytometer. Viability was determined by trypan blue exclusion. For drug testing, only preparations with >95% viability were used. DNA content of the harvest cells was determined by a microfluorometric method (*2, 5*), and DNA synthesis was measured by the method of Michelopoulos et al. (*7*) and expressed as cpm/μg DNA. The hepatocytes were plated at a cell density of 2×10^5/well in a Corning 35-mm tissue culture dish containing 1.5 ml medium and maintained at 37°C in a 5% CO_2 atmosphere. In preliminary viability studies in which the basal medium was enriched with insulin but not with EGF, the viability of these resting hepatocytes over the 48 hr following a 3-hr attachment was diminished only by azathioprine (Table 2).

After a 3-hr attachment period in the definitive in vitro experiments, the medium was replaced with 1.5 ml basal medium (MEM), to which EGF (10 ng/ml) and insulin (10^{-7} M) were added. MPA was dissolved in DMSO, and the other 3 immunosuppressive drugs tested were dissolved in alcohol and added in the concentrations shown in Figure 2. The minute quantities of DMSO or alcohol added to the medium (1 μl/ml) were shown not to affect hepatocyte proliferation (data not shown). During the last half of the 48-hr culture period, 3 μCi ^{3}H-thymidine was added to the wells containing hepatocytes and MP, MPA, or MZ. MZ and MPA inhibited the DNA synthesis of the cultured hepatocytes while the MP caused a near doubling of thymidine incorporation (Fig. 2).

These results and the previously reported ones with cyclosporine, FK506, and RPM provide a basis for further inquiry. They suggest a fundamental difference between the growth effects of cytotoxic agents (AZA, MPA, and MZ) versus previously studied drugs like CsA, FK506, and RPM that are thought to act by altering signal transduction (*8*). The cytotoxic agents act by selectively inhibiting the synthesis of purine nucleotides (adenine or guanine), thereby reducing DNA synthesis of a variety of immunologic and other specialized cells, including hepatocytes. However, in the intact animal, the hepatocyte proliferative response of regeneration was not demonstrably affected by AZA or MPA, although it was drastically reduced by MZ. In contrast, the hepatocytes in vitro were killed (AZA) or rendered nonproliferative (MPA and MZ). Whether the striking effect of MZ in both testing conditions was a high-dose phenomenon or has a more specific explanation could not be determined from our experiments.

The in vivo results that we obtained with a 4-day course before hepatectomy of either 1 or 6 mg/kg/day of azathioprine were remarkedly different than those reported by Kim et al. (*9*) who observed striking augmentation of DNA synthesis in essentially the same experiments. They interpreted this as evidence that hepatic regeneration is immune-modulated. Be-

cause of the possibility that Kim (*9*) might have interposed a delay between the last drug dose and the partial hepatectomy, thereby allowing the beginning of regeneration in response to a chemical injury, we performed more experiments in which the last azathioprine dose was not given at the time of operation. Although the animals with the premature cessation of AZA treatment tended to have more vigorous regeneration (Table 3), this difference was not statistically significant. However, the possibility of a "timing" artefact has not been ruled out and will require further study.

Unlike AZA and the other cytotoxic drugs (MPA and MZ), MP caused a striking increase of proliferation of hepatocytes in culture and it augmented liver regeneration in the intact animal. Such steroid-stimulating effects on hepatocyte division have not been noted before—and, in fact, Richman et al. (*10*) reported inhibition by dexamethasone, which in our model also was stimulatory (unpublished observations). MP is known to inhibit the synthesis and expression of multiple cytokines including IL_1, IL_2, and migration inhibitor factor (*11*, *12*). Although IL_1 and IL_2 are thought from reported in vitro experiments to be growth suppressors (*13*) this could not be demonstrated by us in a sensitive in vivo test system in which the recombinant cytokines in question were infused directly into the tied-off portal vein of the Eck fistula liver (*14*). Thus, the proliferative response to MP reported herein both in vivo and in vitro cannot be explained by what is currently known about steroid actions.

In contrast, the inhibition of hepatocyte proliferation by cytotoxic drugs is consistent with previous reports about AZA (*15*). A seemingly obvious explanation could be that AZA, MPA, and MZ selectively inhibit synthesis of purine nucleotides (adenine with AZA and guanine with MPA and MZ) that are required for DNA synthesis.

The molecular basis of growth alterations by immunosuppressants is best understood with FK506, CsA, and RPM—drugs that alter signal transduction with pleiotropic effects not limited to immune cells (*3*, *8*). These agents, which are not inherently cytotoxic, are inactive "prodrugs" until they bind with ubiquitous cytosolic immunophilins and form complexes that act upon a common target(s) (*8*). Although these molecular mechanisms have been determined principally with the lymphocyte and mast cell, the hepatocyte may be the best to investigate growth control (*6*).

As the search continues for immunosuppressive drugs, it is self-evident that growth control qualities should be systematically determined. Conversely, drugs under study primarily for their hepatocyte growth control activity (*14*) should also be screened for possible immunosuppressive activity.

TABLE 3. Effects on hepatocyte proliferation of AZA administration

Group	n	Operation	AZA administration	Route	cpm/mg DNA
1	5	Sham	1.5 mg/kg for 3 days	PO	4.477±420
2	5	70% PH	1.5 mg/kg for 3 days	PO	87.574±18.315
3	5	70% PH	1.5 mg/kg for 4 days	PO	55.318±15.422

[1] Groups 1 and 2 were treated for 3 days but did not receive AZA on the day of the operation as in our experiments shown in Figure 1 (group 3 in this table).

A. AZZARONE[2,3]
A. FRANCAVILLA[2,3]
Q. H. ZENG[2]
T. E. STARZL[4]
The Department of Surgery
University Health Center of Pittsburgh
Pittsburgh, Pennsylvania
The Department of Gastroenterology
University of Bari
Bari, Italy

[2] University Health Center of Pittsburgh.
[3] University of Bari.
[4] Address requests for reprints to Thomas E. Starzl, M.D., Ph.D., Department of Surgery, 3601 Fifth Ave., University of Pittsburgh, Pittsburgh, PA, 15213.

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