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Treatment of rat nephrotoxic nephritis. Use of 5-fluorouracil or methotrexate-5-fluorouracil association

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SUMMARY

To evaluate the effect of 5-fluorouracil (F) and methotrexate-5-fluorouracil association (MTX-F) on nephrotoxic nephritis, seven groups of 10 rats were inoculated with anti-rat glomerular basement membrane serum (AGBMS); five groups were treated with different doses of F, beginning on the 2nd or the 6th day, one group with MTX-F beginning on the 2nd day and one group (control) with distilled water. Twenty-four hour proteinuria was determined weekly until the 71st day. The kidneys were examined histologically and by immunofluorescence. The group treated with F (1·3 mg/100 g body weight) developed a severe glomerulonephritis similar to the control group; (b) the groups treated with F (2·0 mg/100 g body weight) or with MTX-F showed progressively lower proteinuria, less severe histological changes and less intense fluorescence due to autologous antibodies. The best results were observed in the MTX-F group and in the F group treated from the 6th day. These groups presented at the 71st day proteinuria of 84 and 91 mg as compared to 312 mg in the control group, and minimal histological lesions as compared to glomerulosclerosis and tubular atrophy in the control group. We concluded that either F or MTX-F produced significant improvement of nephrotoxic nephritis due to inhibition of autologous antibody production.

Keywords 5-fluorouracil methotrexate-5-fluorouracil nephrotoxic nephritis

INTRODUCTION

Nephrotoxic nephritis, although caused by heterologous anti-glomerular basement membrane antibody, is an experimental model in which the importance of autologous antibody in the aggravation and maintenance of the disease has been recognized (Kay, 1940; Hammer & Dixon, 1963; Unanue & Dixon, 1965; Gang & Kalant, 1970). Measures that prevent formation of autologous antibody stop the evolution of nephritis (Kay, 1940; Hammer & Dixon, 1963). Thus, this experimental model is useful in studies on the control of nephritis by immunosuppression.

Methotrexate (MTX) and 5-fluorouracil (F) are anti-neoplastic drugs with immunosuppressive activity proven experimentally and in man (Berenbaun, 1975). Heppner *et al.* (1974) employing F or the association of MTX and F (MTX-F), were able to inhibit humoral immunity in mice while preserving cell-mediated immunity.

Humoral immunosuppression without reduction of cell-mediated immunity should be adequate treatment for both nephrotoxic nephritis and human glomerulonephritis.

The present study is an attempt to evaluate the action of F alone or in combination with MTX on nephrotoxic nephritis in rats.

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MATERIALS AND METHODS

Animals. Wistar male rats weighing 183–228 g were used.

Nephrotoxic sera. Nine rabbits were first innoculated s.c. with 10 mg of glomerular basement membrane (GBM), obtained as described by Greenspoon & Krakower (1950), in 1 ml of Freund's complete adjuvant and then inoculated i.p. weekly with 10 mg of GBM in 1 ml of saline and bled 1 week after the 6th inoculation. The pool of the nine rabbit anti-rat glomerular basement membrane sera (AGBMS) and pooled normal rabbit sera (NS) were inactivated for 30 min at 56°C and then stored at −20°C. The content of anti-rat GBM antibodies in rabbit serum was determined by indirect immunofluorescence in isolated rat GBM (Almeida & Franco, 1971, 1976). The AGBMS titre was 1/120, NS was negative.

Induction of nephritis. Six groups of 10 rats were inoculated with 1 ml AGBMS/100 g body weight and one group was inoculated with NS (1 ml/100 g body weight).

The rats were kept in metabolic individual cages and fed, *ad libitum*, ordinary rat food and water. Twenty-four hour urine was collected on the 1st day after AGBMS injection and weekly thereafter, to evaluate proteinuria.

Urine protein. Urine protein was assayed by the biuret method.

Plasma creatine. Rats were sacrificed on day 71 and their blood collected for plasma creatinine determination by creatine "Labtest" kit (Cat no. 35-Labtest S.A.-São Paulo-Brasil).

Treatment (Table 1). Doses of F and MTX-F were the same as used by Heppner *et al.* (1974). The 2nd day was selected for administration because it preceded the onset of the autologous stage of nephritis, and the 6th day because it coincided with the onset of this stage. The programs of maintenance were selected among those used with these drugs (Calabresi, 1980).

Five groups were treated with F i.p., group 1 rats were inoculated with 1 ml/100 g of body weight of a saline solution (SS) containing 1·3 mg/ml of F. Rats in groups 2, 3, 4 and 7 were treated with similar solution containing 2 mg/ml F.

Group 5 were treated with solutions of MTX and F, administered in that order with a 1 h interval. Each rat was injected i.m. with 0·5 ml/100 g body weight of SS containing 0·1 mg MTX, and i.p. with 1 ml/100 g body weight of SS containing 5 mg F.

Histological study. The lower half of the right kidney of each rat was fixed in Hely's, embedded in paraffin, cut into 4 μm sections, and stained with HE and PAS.

To compare the degree of renal lesions, the alterations specified in Table 2 were quantified (+ to + + + +) as proposed by Almeida & Franco (1976). The total number of signs attributed to each of glomerular alterations was used as index of glomerular lesion (IGL). As index of tubular lesion (ITL) we used the sum of crosses of tubular alterations. Index of renal lesion (IRL): was the sum of IGL ITL and intersticial alterations.

Renal lesions were evaluated without knowledge of rat group or number.

Immunohistochemical study. The left kidney of each rat was frozen in liquid nitrogen and stored at −20°C; 4 μm sections were cut and stained with fluorescein conjugated anti-rabbit gammaglobulin (Hyland Division Travenol Lab), anti-rat gammaglobulin (Hyland), anti-rat C3 (Cappel Lab) and anti-rat fibrinogen (Cappel Lab) by the method of Coons & Kaplan (1950); the slides were examined under a Zeiss Standard RA fluroescence microscope with a BG/2 excitor and a 50 barrier filter. The intensity of immunofluorescence was graded from 0 to 4 by the following criteria: 0 = negative fluorescence; + = faint linear fluorescence of GBM or occasional foci of fluorescent granularity; + + = linear fluorescence of GBM without intense brightness or many foci of fluorescent granularity; + + + to + + + + = intense brightness of GBM or bright fluorescent granularity in almost all glomeruli.

Experimental protocol. In pilot experiments, MTX-F did not increase protein levels or altered renal histology in normal rats, and for this reason we decided to use a single control group of normal rats treated with the highest total dose of F. Table 1 describes experimental protocol.

Statistical analysis. Proteinuria and creatinine were only evaluated in rats surviving up to the 71st day. (1) Proteinuria. The values were transformed using the $Y = \log (x + 100)$ ratio (Y = transformed variable; x = measured proteinuria). (a) Proteinuria on the 1st day after

inoculation: 1st day proteinuria levels were submitted to a fixed model fully randomized analysis of variance followed by multiple comparisons of differences between means by the Tukey test (Steel & Torrie, 1960), ($\alpha = 0{\cdot}05$). (B) Proteinuria during the experiment: proteinuria in various groups throughout the experiment was tested by profile analysis (Morrison, 1967). The hypotheses were tested by the 'F' parameter, $\alpha = 0{\cdot}01$.

(2) Creatinine. The data were submitted to fixed model fully randomized analysis of variance. Differences between means we used for multiple comparison by the Tukey test (Steel & Torrie, 1960), $\alpha = 0{\cdot}05$.

(3) Indices of glomerular, tubular and renal lesions. The data were submitted to a non-parametric analysis of variance by the Kruskal–Wallis method (Colquhoun, 1971).

Levels of significance are indicated in Table 2. Seven rats from each group were selected at random among those that survived until day 71 and used for analysis of the data.

RESULTS

Survival

Table 1 shows numbers of rats surviving up to day 71. Others animals died in the first 2 weeks as a result of both acute proliferative glomerulonephrites and drug toxicity.

Proteinuria

Fig. 1 shows means of the weekly proteinuria for each group of rats surviving until the day 71. All animals receiving AGBMS had proteinuria starting on the first day of the experiment.

From day 15 on, the nephritic control group had a small and persistent increase in proteinuria.

Other groups showed a reduction in levels, with small oscillations in groups 1, 2, 3 and 5, and sharp increases and decreases in group 4. Decreases ocurred following medication.

Rats receiving nephrotoxic serum had similar proteinuria on the first day of experiment; they were significantly higher than those of the NS inoculated group ($P < 0{\cdot}05$).

When groups 1, 2, 3, 4, 5 and 6 were compared day by day, differences in proteinuria only become statistically significant ($P = 0{\cdot}001$) only from day 29 on. Despite these differences for the groups as a whole, groups 3 and 5 behaved similarly during this period and differed from group 6.

Plasma creatinine

Groups 1–6 did not differ amongst themselves in creatinine levels or from a group of 10 normal rats

Table 1. Experimental protocol

Group	Inoculum at 1st day	Starting day	Treatment		Rats surviving at day 71
			Drug	Days of drug administration	
1	AGBMS	2	F(1·3)	2 to 6–16–23–30–37–44–51–58–65	9
2	AGBMS	2	F(2·0)	2 to 6–16–23–30–37–44–51–58–65	7
3	AGBMS	6	F(2·0)	6 to 10–18–25–32–39–46–53–60–67	8
4	AGBMS	2	F(2·0)	2 to 6–23 to 27–44 to 48	7
5	AGBMS	2	MTX-F	2–32	7
6	AGBMS	2	W	2 to 6–23 to 27–44 to 48	9
7	NS	2	F(2·0)	2 to 6–23 to 27–44 to 48	9

AGBMS = anti glomerular basement membrane serum; NS = normal serum; F(1·3) = 5-fluorouracil (1·3 mg/100 g body weight); F(2·0) = 5-fluorouracil (2·0 mg/100 g body weight); W = distilled water; MTX-F = methotrexate (0·1 mg/100 g body weight) and 1 h later 5-fluorouracil (5·0 mg/100 g body weight).

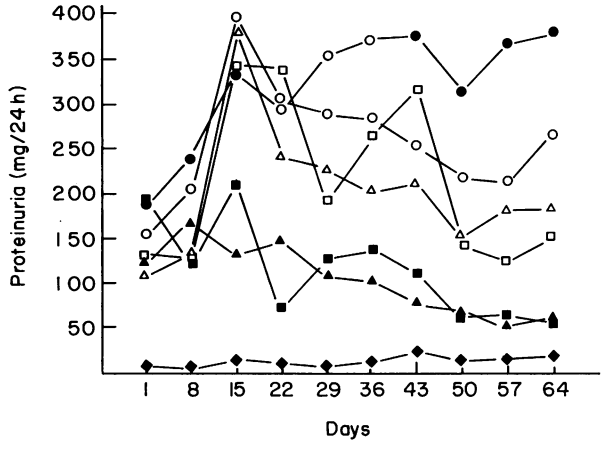

Fig. 1. Proteinuria, ○——○ group 1; △——△ group 2; ▲——▲ group 3; □——□ group 4; ■——■ group 5; ●——● group 6; ♦——♦ group 7.

of the same age. Group 7 (1·18 ± 0·22) differed significantly from group 3 (0·88 ± 0·33) and from normal rats (0·88 ± 0·11).

Histology

Kidneys of treated normal animals (group 7) were histologically normal. Lesions of membranoproliferative glomerulonephrites as previously described (Almeida & Franco, 1976) were found in all other rats of the remaining groups. Intensity of histological renal alterations in groups 1 to 6 are shown in Table 2. The indexes of glomerular and renal lesions for groups 3 and 5 were smaller and differed from those for group 6 ($P < 0·05$), the remaining differences being non-significant. On rats of group 3 IRL values were low with the exception of one rat in which it reached 38. The pattern of lesions for rats whose IRL where the highest in groups 5 and 6 are shown in Fig. 2.

Immunohistochemistry

Rabbit gammaglobulin was not found in kidneys of group 7 rats but was always present, in a diffuse linear pattern, only in the GBM of rats of other groups (Fig. 3). No difference in fluorescence pattern or intensity was detected between groups.

Table 2. Means of glomerular and tubular histological alterations. Index of glomerular (IGL), tubular (ITL) and renal (IRL) lesions (means)

	Glomeruli										Tubules				Intersticium		
Group	IS	L	AH	PH	S	MMI	GBM	CH	TH	IGL	D	R	A	ITL	II	F	IRL
1	2·1	1·6	1·5	3·1	3·2	2·1	2·2	2·5	2·3	2·1	2·4	1·4	2·0	5·8	1·6	0·7	29·2
2	2·1	1·9	1·4	2·4	2·1	1·9	1·4	1·1	1·4	15·8	1·7	0·8	1·0	3·5	1·1	0·3	20·8
3	1·0	1·1	1·0	1·1	1·1	1·2	1·1	0·5	0·8	8·9†	0·7	0·4	0·5	1·6	0·3	0·2	11·0†
4	1·7	1·6	1·4	2·7	2·3	1·7	1·6	1·1	1·6	15·7	1·6	1·1	1·3	4·0	1·0	0·3	20·8
5	1·3	1·4	1·6	1·7	1·5	1·4	1·0	0·8	1·0	11·7*	1·1	0·4	0·2	1·7	0·8	0·0	14·2*
6	3·1	2·1	1·3	3·6	3·5	2·5	2·4	3·2	3·4	25·1	2·5	1·9	2·0	6·4	2·1	1·0	34·6

IS = increase in size; L = lobulation; AH = axial hypercellularity; PH = parietal hypercellularity, S = synechiae; MMI = mesangial matrix increase; GBM = thickening of GBM; CH = capsule hyalinization; TH = hyalinization of glomerular tuft; D = degeneration; R = regeneration; A = atrophy; II = inflammatory infiltrate; F = fibrosis. Asterisks indicate significant differences from group 6, control, (*$P < 0·05$; †$P < 0·01$).

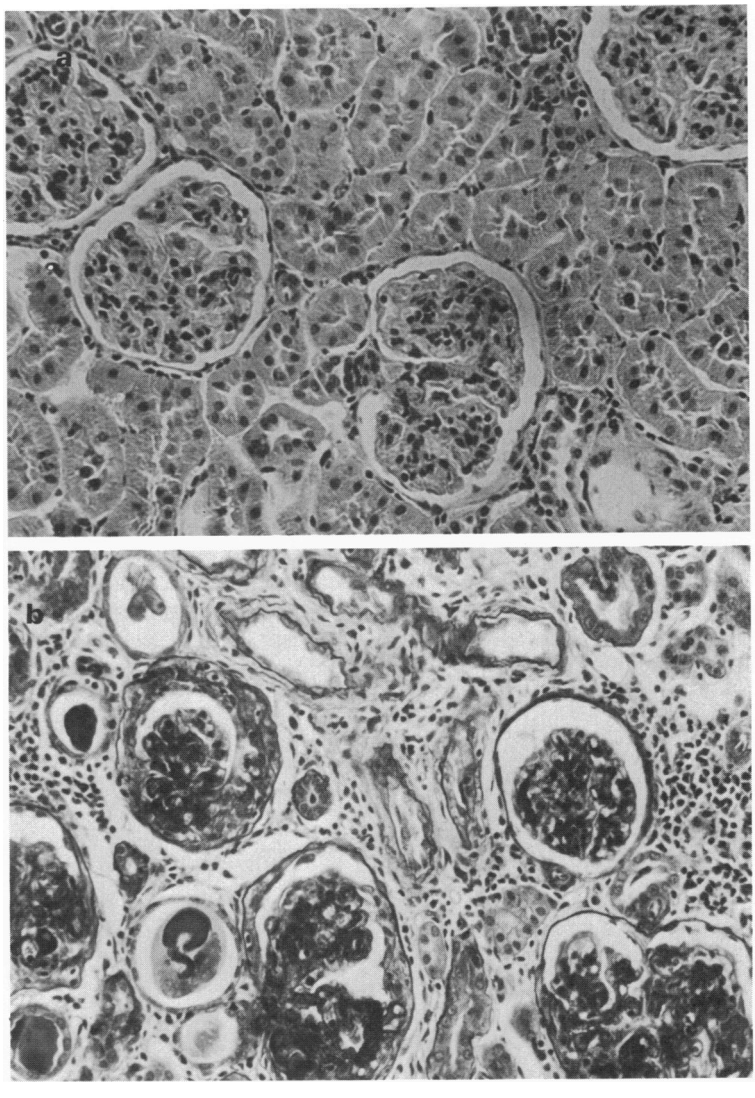

Fig. 2. Histological aspect of kidneys of rats from groups 5 and 6 whose IRL were the highest in their corresponding groups IRL; (a) rat 5.7, IRL = 18 (× 160); (b) rat 6·7, IRL = 46 (× 160). Note that rat 6·7 presented more intense glomerular alterations than rat 5·7.

Rat gammaglobulin was not found in the kidneys of group 7 rats but was found, in a diffuse linear pattern, only in the GBM of all rats of the other groups, with the exception of group 5. Bright fluorescence was observed in the glomeruli of group 6 (Fig. 3) in relation to all other groups and this difference being greater between groups 5 and 6. In group 5, rat gammaglobulin was not found in the renal tissue of three rats. whereas the others exhibited skipped and low intensity fluorescence in the glomeruli (Fig. 4).

C3 was only detected, in a linear pattern, in the GBM of animals from groups 1 and 6. Tubular basement membrane fluorescence of low intensity was observed in all groups. Fibrinogen was present in a granular pattern in the capillary loops and tubular basement membranes (TBM) of only one rat in group 5, whereas fluorescence was positive and usually of medium intensity in the glomerular loops of all animals, in the capsule of six, and in the TBM of three animals in group 6 (Fig. 4).

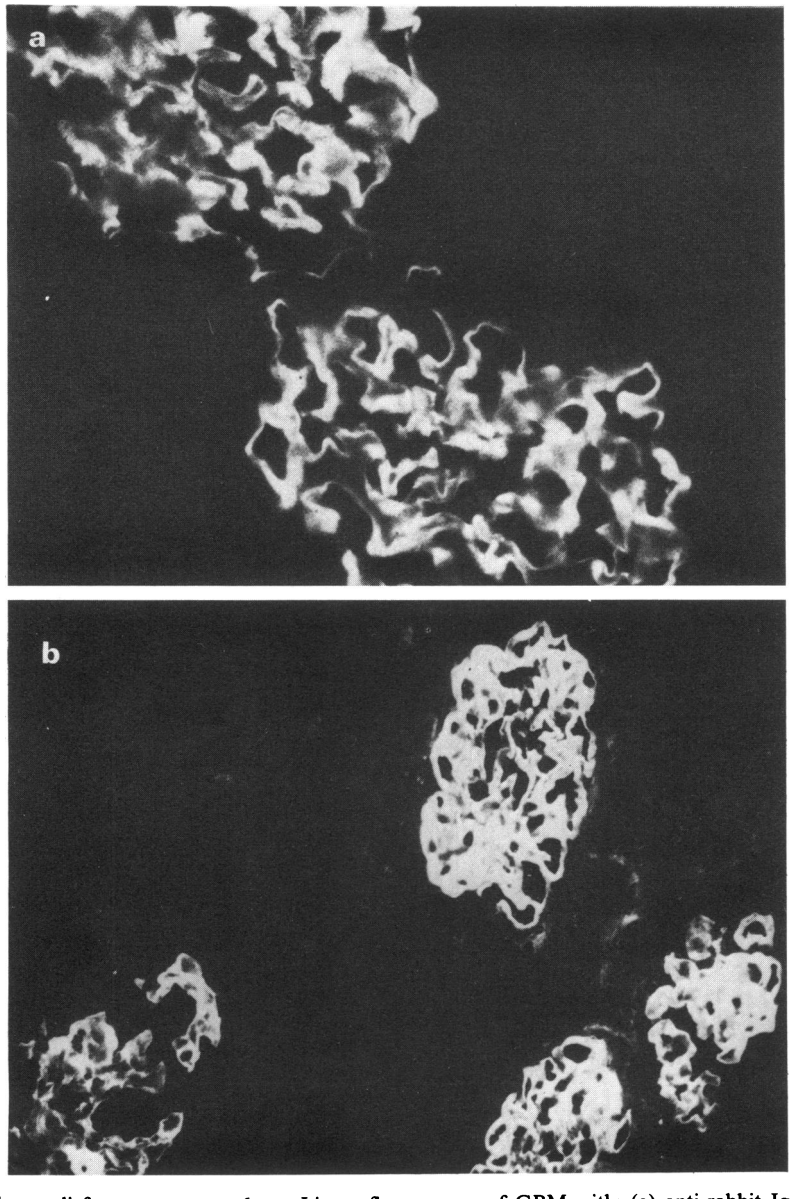

Fig. 3. Glomeruli from one group 6 rat. Linear fluorescence of GBM with: (a) anti-rabbit Ig fluorescein conjugated antiserum; (b) anti-rat Ig fluorescein conjugated anti-serum.

DISCUSSION

The least toxic treatment was F at the dose of 1·3 mg/100 g body weight.

The increase in proteinuria in group 7 rats did not exceed the levels expected for ageing rats (Perry, 1965) and in them no histological lesions were observed.

The similar proteinuria shown by AGBMS inoculated groups on the 1st day suggests that all groups received equivalent amounts of anti-GBM antibody and developed nephritis of similar intensity.

Proteinuria observed in all the AGBMS inoculated groups, indicate that all developed the autologous phase of nephrotoxic nephritis.

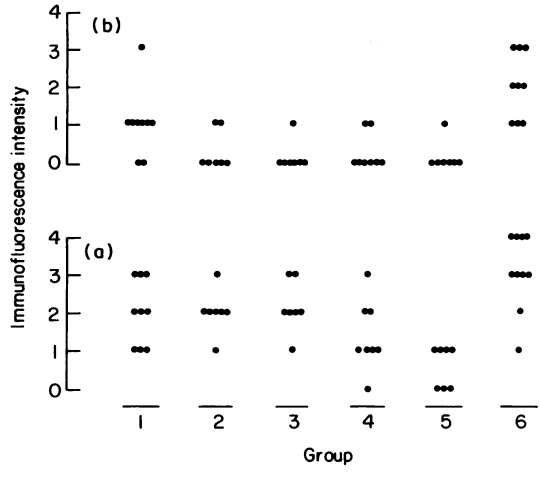

Fig. 4. Intensity of glomerular immunofluorescence staining for: (a) rat Ig and (b) rat fibrinogen.

The lack of increase in proteinuria between 8th and 15th day in group 3, the intensity of this increase in group 5, insufficient to exceed the proteinuria levels observed on the 1st day and the slowly decrease of proteinuria from 15th day on, in group 3 and 5, suggests a partial and persistent blockade of the production of autologous antibody in these groups. The tendency towards a small and progressive decrease in proteinuria observed in groups 1 and 2 from the 15th day on, indicates that a certain inhibition in formation of autologous antibody occurred from the 15th day on. The marked and transitory reduction in proteinuria observed in group 4 at the time of administration of the intermittent F series suggests that the production of autologous antibody was strongly but transitorily inhibited.

Drug administration in groups 2 and 3 was similar, except for starting time; proteinuria and IRL were lower in group 3 than in group 2 indicating that F, administered at the beginning of autologous phase, is more effective in inhibiting antibody production than when administered earlier. The degree of immunosuppression exerted by F, is more intense at the time when immunocompetent cells are in proliferation (Schwartz, 1965); F exerts immunosuppression only when administered after antigen inoculation (Berenbaun, 1979).

In group 3, six rats presented IRL equal to or lower than 12, one had IRL 38, indicating greater damage. We assume that, F had no immunosuppressor effect on this animal. Irregularity of the suppressing action of F in humoral immunity has been reported by Heppner *et al.* (1974).

The schemes of F and MTX-F used in groups 3 and 5 greatly reduced renal lesions.

The results of immunofluorescence suggest that F and the MTX-F association inhibit autologous antibody production.

In group 3 (Fig. 4), rat gammaglobulin fluorescence was similar to group 2 and more intense than in group 5. This contradict the hypothesis that immunosuppression was more effective with the scheme used in group 3 than in groups 2 and 4. However, in group 3 treatment was initiated on the 6th day. Autologous antibody appears in glomeruli on the 4th day (Shigematsu & Kobayashi, 1971) after inoculation of nephrotoxic serum, its half-life in the kidney being 17 days (Unanue & Dixon, 1965). By the time treatment was initiated in group 3, autologous antibody was already fixed on the kidney being at least partly responsible for the fluorescence. From this moment on the inhibition of autologous antibody production exerted by the drug made available none or only small amounts of this antibody and determined the reduction of renal lesions when compared with nephritic control group.

The low fluorescence observed with anti-complement immunoglobulin, complicates its evaluation.

Drugs given to treated nephritic groups inhibited fibrinogen deposition on the glomeruli,

especially on Bowman's capsule, related, by Vasalli & McCluskey (1964) to the formation of crescents.

The large number of crescents observed in nephritis induced by nephrotoxic sera with high titres of kidney fixing antibody (Holdsworth *et al.*, 1978) as well as by previous immunization with heterologous globulin (Bone *et al.*, 1975) are evidence for the relation between crescent and amount of heterologous or autologous antibody fixing on the kidney. These results and these of Nakamoto *et al.* (1978) demonstrate that the change in coagulation is secondary and dependent on the immunological phenomenon.

For these reasons, one may suggest that the small fibrinogen's deposition in treated animals is an indirect sign of the smaller deposition of autologous antibody, and therefore, of the immunosuppression exerted by F and MTX-F.

The differences between groups treated from the 2nd day on and the nephritic control group were significant only between control and group treated with MTX-F. However, the reduced renal lesions in F medicated groups treated from the 2nd day on although not significant, indicate that an increase in sample or in duration of the experiment may demonstrate the action of these treatments on the intensity of nephrotoxic nephritis.

We conclude that: (a) the administration of MTX-F association from the 2nd day of nephrotoxic nephritis on, reduced the intensity of the disease; (b) F administered from the 6th day on at the dose of 2·0 mg/100 g body weight/day, was as effective as the MTX-F association; (c) F was more effective when first administered on the 6th day.

However further studies are needed to determine if the positive treatment effect observed in group 5 was due to the high single dose of F alone or to the presence of MTX.

As to the mechanism of action of F and MTX-F, the results suggest that MTX-F and F, administered after the inoculation of nephrotoxic serum, inhibit the production of autologous antibody and, secondarily, the intravascular coagulation and spillage of fibrinogen into Bowman's capsule.

Similar results were obtained when the MTX-F association was administered from the 8th or 28th day on to nephritic rats (unpublished).

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