Action of D-penicillamine on immunocomplexes containing rheumatoid factor

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Summary. The affinity between purified rheumatoid factors (RF) and native or heat aggregated human IgG has been studied in vitro by polarization florescence in the presence and in the absence of D-penicillamine. The value of the dissociation constant was the same using native and heat aggregated IgG suggesting that binding to the aggregated protein is not dependent on the exposure of a new determinant lacking in the native molecule. The results obtained in the presence of D-penicillamine suggest that the concentration of the drug necessary to get a pronounced effect on the apparent dissociation constant of the immunocomplex between IgG and RF is not reached in vivo, in clinical situations.

It has been shown that D-penicillamine therapy in rheumathoid arthritis leads to a reduction in the level of circulating soluble immune complexes in which rheumatoid factor is a component[1]. 2 mechanisms have been proposed for this action of the drug. One is that it acts as a general immunosuppressive agent[2]. A 2nd mechanism may be that D-penicillamine dissociates immune complexes by forming mixed disulphides with protein[3].

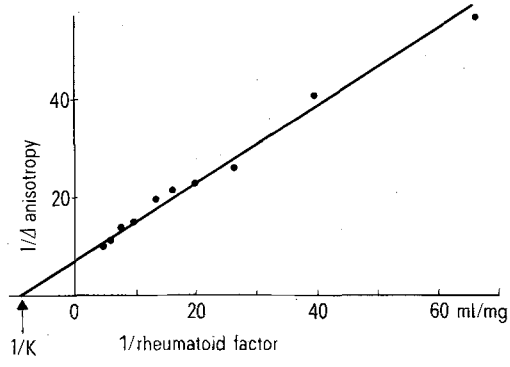
In the present paper, the effect of D-penicillamine on the affinity between purified rheumatoid factors (RF) and human IgG has been studied in vitro by fluorescence polarization measurements. This very sensitive technique makes it possible to work at very low concentrations of IgG and in a molar excess of RF. Under these conditions, protein aggregation, which is a consequence of the multivalent nature of the pentameric IgM RF molecules, can be prevented.

Materials and methods. RF, purified from the serum of a patient with classical rheumatoid arthritis by affinity chromatography[4], had the following immunoglobulin composition measured by quantitative immunodiffusion[5]: 8% IgA, 23% IgG, 69% IgM. These immunoglobulins accounted, within the experimental error, for the protein content of the purified RF measured spectrophometrically at 280 nm[6].

IgG, purified from human serum by DEAE cellulose (Whatman) chromatography[7], was labelled with fluorescein isothiocyanate[8]. The excess of dye was completely removed by Sephadex G 25 (Pharmacia) filtration as demonstred by gel-electrophoresis[9]. The degree of labelling, determined spectrophotometrically[10], was 4 ± 0.5 moles of dye per mole of IgG. The intensity and polarization fluorescence and the anisotropy of labelled IgG were calculated as previously described[11]. All experiments were carried out at 25 °C in phosphate buffered saline, pH 7.4, containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter.

Results and discussion. Fluorescence polarization of labelled IgG (4.8×10^{-3} mg/ml) was measured in the presence of increasing concentrations of purified RF (ranging from 1.5×10^{-2} mg/ml to 0.23 mg/ml). Each measurement was carried out as soon as a constant fluorescence polarization value was obtained (usually after 1 min from the addition of RF to labelled IgG). Fluorescence polarization of labelled IgG increased from 0.11 to a limit value of 0.25 in the presence of increasing concentrations of RF. Control experiments showed that fluorescence polarization of labelled IgG was not affected by the addition of human serum up to 0.5 mg/ml.

The quantum yield of the fluorescein conjugated IgG was not significantly influenced by the addition of RF. Therefore the concentration of the immune complex was proportional to the difference between the anisotropy measured in the presence of RF and that determined in absence of RF. The value of the dissociation constant ($K = 0.12$ mg/ml) was calculated from the double reciprocal plot shown in the figure.



Double reciprocal plot of anisotropy variation of labelled IgG versus concentration of purified rheumatoid factor. The experimental conditions are described in 'materials and methods'.

The value obtained is of the same order of magnitude as the one reported by other investigators using different techniques[6]. Furthermore, in agreement with other reports[6,7], the value of the dissociation constant was the same both with native and heat aggregated IgG, confirming that the binding with the aggregated protein is not dependent on the exposure of a new determinant lacking in the native molecule.

The value of the apparent dissociation constant of the RF-labelled IgG complex was not appreciably affected in the presence of D-penicillamine up to 5×10^{-4} M, when the drug was incubated at 25 °C with the immune complex for 24 h. To observe a 70% decrease in the concentration of the immune complex it was necessary to use 5×10^{-2} M D-penicillamine with an incubation time at 25 °C of at least 2 h.

Taking into account that in rheumatoid patients receiving 750 mg of D-penicillamine daily a drug level lower than 5×10^{-5} M is reached in plasma[12] and that immune complexes generally have a relatively short intravascular half life[13,14], it seems unlikely that the concentration of D-penicillamine necessary to dissociate immune complexes between IgG and RF is reached in clinical situations.

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Inhibitory effect of galactose on hydrogen transfer in the hemolymph of *Vespa orientalis*

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Summary. The generation of pyruvate and lactate in *Vespa orientalis* hemolymph is prevented by the presence of galactose. The inhibitory effect is possibly produced by a competition for NAD and occurs in concentrations of 0.05 M.

Galactosemia is a well-known disease state brought about by an inborn metabolic error. In man and experimental animals such excess galactose may produce a variety of symptoms ranging from mild to fatal. Its clinical effect and the putative biochemical defects are discussed extensively by Isselbacher[1]. The disease is presumably due to an absence of the enzyme galactose-1-phosphate uridyl transferase, and results in an accumulation of galactose and galactose-1-phosphate, neither of which is toxic in the normal state. In galactosemia, unlike in other enzymatic disorders involving dysfunction of a single enzyme, a large number of other enzymes are inhibited[2].

In a previous investigation of monosaccharidase activity in the hemolymph of *Vespa orientalis*[3] we found that while glucose and fructose are freely utilized via both the aerobic and anaerobic pathways, galactose is not, and its presence interferes with the oxidation of these 2 monosaccharides, as measured in the enzymatic assay[4]. It was further found that hornet larvae die when fed on galactose[5].

Materials and methods. Hemolymph was obtained from the 'dark pupae' (i.e., pupae before eclosion) of *Vespa orientalis.* The reaction was carried out at pH 7.6 using 0.01 M phosphate buffer. Starting values were established prior to the incubation, and any changes in both the lactic and pyruvic acid levels were determined at 60 min post incubation, using Varley's method[4] for the enzymatic assay.

Final volume of the reaction mixture was 5 ml, consisting of 2.5 ml buffer, 2.0 ml of galactose solution prepared in 6 increasing concentrations from 0 to 0.1 mM/ml, and 0.5 ml hemolymph. Lactate and pyruvate levels in each sample prior to incubation were taken as zero and the monosaccharidase activity, measured as described in our previous work[3], was expressed in terms of μM of lactate and pyruvate/ml present after 60 min incubation at 37 °C. The inhibition effect of galactose on lactate and pyruvate production could thus be assessed.

Results. A total inhibition of lactate production was observed in galactose concentrations of 0.05 mM/ml and above, and a partial inhibition of pyruvic acid generation – at galactose concentrations of 0.5 mM/ml and above. The results are summarized in the table.

Discussion. In a previous study we demonstrated monosaccharidase activity in *Vespa orientalis*[3], encountering oxidation both by the aerobic and anaerobic pathways, but not in the presence of galactose. Apparently, galactose blocks the anaerobic (lactate) pathway of monosaccharide utilization even in low concentration whereas in higher concentration it also blocks the aerobic (pyruvate) pathway. These findings suggest several possibilities. First, that galactose not only cannot be utilized by *Vespa orientalis* larvae and pupae (and probably by various other insects) but may prove to be deadly poisonous for them. As such and because it is ordinarily absolutely harmless to all other organisms galactose should comprise an effective pesticide-insecticide, superseding ecologically dangerous DDT and the potentially lethal organo-phosphates. Its practicability, however, has yet to be proven by actual field trials.

Secondly, the results of our present study suggest also that, among the congenital metabolic defects (enzyme deficiencies), galactosemia causes the widest biological damage because it interferes with most of the enzymatic reactions dependent on the coenzyme NAD-NADH, that is, with most of the hydrogen transfer reactions. Whether this is due to competition or actual inhibition has yet to be established.

Inhibition of monosaccharidase activity in the hemolymph of *Vespa orientalis* larvae by the presence of galactose

Monosaccharides added	Galactose concentration (mM/ml) 0.00		0.01		0.05		0.20		0.50		1.00	
	L	P	L	P	L	P	L	P	L	P	L	P
Fructose 1 mM/ml	450	1900	110	1650	0	200	0	144	0	±	0	0
Glucose 1 mM/ml	140	950	20	200	0	25	0	±	0	0	0	0

L, μM lactate/ml, produced at 37 °C in 60 min; P, μM pyruvate/ml, produced at 37 °C in 60 min.

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