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The mode of action of treatment by IgG of haemolytic anaemia induced by an anti-erythrocyte monoclonal antibody

Y. POTTIER, I. PIERARD, A. BARCLAY*, P. L. MASSON & J.-P. COUTELIER *Unit of Experimental Medicine, International Institute for Cellular and Molecular Pathology, Université Catholique de Louvain, Bruxelles, and *Baxter Inc., Nivelles, Belgium*

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SUMMARY

In order to gain insight into the mechanisms by which the infusion of IgG can improve some autoimmune diseases, we induced haemolytic anaemia in mice by the injection of anti-erythrocyte MoAbs derived from NZB mice by S. Izui (Geneva). The IgG1 antibody 31-9D induces anaemia by erythrocyte sequestration in the spleen and liver, whereas the IgG2a antibody 34-3C triggers erythrophagocytosis (Shibata *et al.*, Int Immunol 1990; **2**:1133). Treatment of mice with pools of either human or mouse IgG clearly attenuated the anaemia induced by 34-3C, but not by 31-9D. Similar protection was obtained with human monoclonal IgGs from myeloma patients. Prior absorption by mouse erythrocytes did not affect the efficacy of the injected IgG. Treatment with Fc fragments also reduced the anaemia. *In vitro* experiments confirmed that 34-3C, but not 31-9D, triggered erythrocyte phagocytosis by murine macrophages. This process was completely inhibited by addition of polyclonal or myeloma IgG or of human Fc fragments. These results indicate that, in this model of autoimmune pathology, the protective effect of IgG is mediated by its interaction with the macrophage Fc receptors.

Keywords immunoglobulin autoimmune haemolytic anaemia phagocytosis macrophage Fc receptor

INTRODUCTION

It is now well established that the administration of IgG (IVIg) can result in clinical improvement in most patients with certain autoimmune disorders, particularly immune thrombocytopenia, Kawasaki syndrome or Guillain–Barré syndrome [1–6]. Less striking beneficial effects have also been reported in several other autoimmune diseases, including myasthenia gravis; most, but not all of these diseases are mediated by autoantibodies [1,7–9]. Even in patients with a similar pathology, the therapeutic response can, however, vary widely, illustrating that a precise understanding of the mode of action remains elusive. If the possibility is excluded that contaminants in the IgG preparations are responsible for the therapeutic effect in certain autoimmune conditions, it may be assumed that either the Fc or Fab regions of the IgG mediate the effect (reviewed in [10]). Fab fraction-mediated efficiency of pooled human IgG might be explained by anti-idiotypic activity against autoantibodies [11–16], by modulation of B and T lymphocyte functions through binding to these cells [16–21], by recognition and elimination of infectious agents associated with autoimmune diseases [10,22], or by modulation of the activity of various lymphokines, including IL-1, IL-6 and tumour necrosis factor (TNF) [23–26]. Through their Fc region, IgG could, among other possible mechanisms [7], increase the catabolism of autoantibodies [10], or decrease antibody production, via the production of soluble Fc receptors [27–31]. However, one of the most probable mechanisms that could explain the therapeutic activity of IgG pools is a temporary blockage of Fc receptors on phagocytic cells.

So far, the lack of suitable experimental models has resulted in limited understanding of the mode of action of pooled IgG. To address this question, we used monoclonal anti-erythrocyte autoantibodies, capable of inducing autoimmune haemolytic anaemia in mice (gift of S. Izui [32,33]). Our results demonstrate that, in this model at least, the success of IgG treatment depends on the Fc-mediated blockage of phagocytic cells.

MATERIALS AND METHODS

Mice

Female BALB/c mice were bred at the Ludwig Institute for Cancer Research by G. Warnier and used when 6–8 weeks old.

IgG

Human IgG was Gammagard (Baxter, Lessines, Belgium). Mouse

Correspondence: J.-P. Coutelier, Unit of Experimental Medicine, ICP, 7430, Av. Hippocrate, 74, B-1200 Bruxelles, Belgium.

IgG was prepared from pooled sera of NMRI mice by affinity chromatography on a protein G column. The pooled IgG was found to be free of bacterial lipopolysaccharide. For *in vivo* experiments, mice usually received five daily doses of 4 mg, which corresponds to the dose received by patients.

Human IgG were adsorbed with mouse erythrocytes by incubating for 1 h at room temperature 100 mg IgG in ≈ 70 μl saline with 210 μl washed mouse erythrocytes.

The preparation of human Fc fragments was adapted from Cerottini [34]. Briefly, IgG was incubated for 2 h at 37°C in PBS with 0·002 M ethylenediaminetetraacetic acid (EDTA)-disodium salt and 0·01 M L-cysteine hydrochloride. IgG concentration was adjusted at 100 mg/ml with 0·15 M NaCl, and 2 mg papain were added per 100 mg IgG. After 15 min at 37°C, the reaction was stopped at 4°C. IgG and Fc fragments were purified by chromatography on Protein A, and Fc fragments were separated by chromatography on an Ultrogel 3.4 column (Pharmacia, Uppsala, Sweden), then dialysed against PBS. The purity of the resulting Fc fragments was analysed by acrylamide gel electrophoresis.

Antibody

31-9D and 34-3C anti-mouse erythrocyte MoAbs, derived from NZB mice, were a kind gift of S. Izui [32,33].

Haematocrit

Haematocrit was measured after centrifugation of heparinized blood in a Hettich-Haematokrit centrifuge (Hettich, Tuttlingen, Germany).

In vitro *erythrophagocytosis*

Sensitized erythrocytes were prepared by incubating 500 μl packed normal erythrocytes with 50 μg MoAb in 10 ml PBS containing 2% bovine serum albumin (BSA) for 30 min at 37°C, then for 1 h at room temperature. Peritoneal cells were collected and allowed to adhere on a tissue culture Petri dish for 3 h. After washing, they were incubated for 16 h at 37°C with 20 μl washed sensitized erythrocytes in 2 ml Dulbecco's minimum essential medium containing 10% decomplemented fetal calf serum (FCS) and supplemented with L-asparagine $0{\cdot}24 \times 10^{-3}$ M, L-arginine $0{\cdot}55 \times 10^{-3}$ M, L-glutamine $1{\cdot}5 \times 10^{-3}$ M, and 2-mercaptoethanol 5×10^{-5} M. As indicated, inhibitory proteins were added during this incubation. Cells were washed with PBS and stained with 0·1% o-toluidine in PBS with 10% FCS.

RESULTS

IgG treatment of passively induced autoimmune haemolytic anaemia

The two anti-erythrocyte MoAbs 31-9D and 34-3C derived from NZB mice have been shown by Shibata and colleagues to induce autoimmune anaemia by separate and distinct mechanisms [33]. Whereas 31-9D of isotype IgG1 causes erythrocyte sequestration in the liver and spleen, 34-3C of isotype IgG2a induces erythrophagocytosis. This difference in mechanisms renders these two antibodies particularly interesting for the study of the therapeutic mode of action of pooled IgG. The MoAbs were injected intraperitoneally into BALB/c mice, which became anaemic 4 days after injection of 1 mg antibody (Fig. 1). To determine whether IgG could alleviate this antibody-induced anaemia, we treated mice every day from day 0 to day 4 with one i.p. injection of 4 mg mouse pooled IgG, a dose per weight corresponding to the immunoglobulin treatment used in human patients. Figure 1 shows the data for a typical experiment, indicating that the treatment significantly reduced the anaemia caused by 34-3C, whereas there was no effect on the anaemia induced by 31-9D. This result suggests that the therapeutic effect is mediated by blockage of phagocytic cells. Similar results were observed upon treatment with polyclonal human IgG (Fig. 2), as well as with human myeloma IgG. This prevention of anaemia was not improved with higher IgG dose (not shown). Polyclonal human IgG preabsorbed with normal mouse erythrocytes also prevented anaemia. Moreover, human Fc fragments also reduced the anaemia, indicating that the therapeutic effect was related to constant regions rather than to anti-idiotypic reactions.

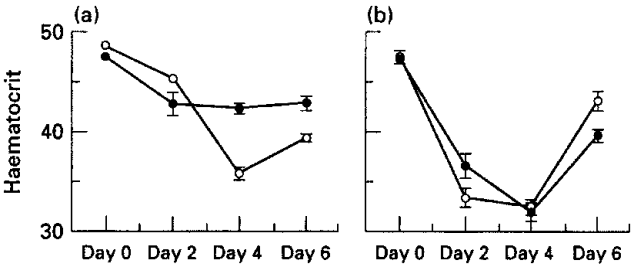

Fig. 1. Effect of mouse IgG treatment on antibody-induced anaemia. BALB/c mice (six per group) received 1 mg of anti-erythrocyte MoAb on day 0. Mouse IgG were injected intraperitoneally at a daily dose of 4 mg. Haematocrit was determined at different times after antibody injection (mean ± s.e.m.). (a) ○, 34-3C; ●, 34-3C + mouse IgG. (b) ○, 31-9D; ●, 31-9D + mouse IgG.

Inhibition of antibody-mediated erythrophagocytosis with total IgG

To analyse further the effect of pooled IgG, we used an *in vitro* model of erythrophagocytosis of sensitized erythrocytes by peritoneal macrophages. As expected, a strong erythrophagocytosis of 34-3C-, but not of 31-9D-coated erythrocytes was observed (Table 1). When macrophages were co-incubated with pooled

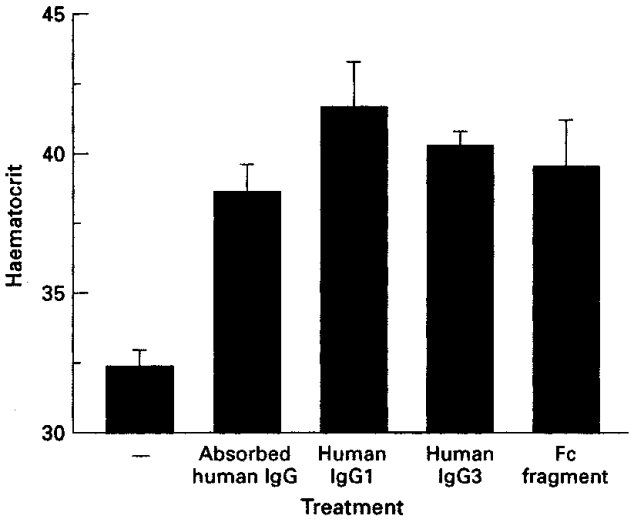

Fig. 2. Effect of various treatments on anaemia induced with 34-3C MoAb. Anaemia was induced in BALB/c mice (four to five per group) by injection of 1 mg 34-3C antibody on day 0. Different treatments were then administered, as indicated, at a daily protein dose of 4 mg. Haematocrit was measured on day 4 (mean ± s.e.m.).

Table 1. *In vitro* inhibition of autoantibody-mediated erythrophagocytosis

Erythrocyte sensitization	*In vitro* treatment	Dose (μg/2 ml)	Erythrophagocytosis (%)*
Nil	–		0
31-9D	–		2
34-3C	–		90–97
	Mouse IgG	400	2
	Human IgG	800	1
	Human IgG	200	0
	Human IgG	50	2
	Human myeloma IgG1	500	0
	Human myeloma IgG3	500	2
	Fc fragments	1000	1
	Fc fragments	250	2

* Percent of macrophages having phagocytized five erythrocytes or more; composite results of five experiments.

human IgG, their ability to ingest 34-3C-coated erythrocytes was completely inhibited (Fig. 3). A similar effect was found with mouse IgG, with human myeloma IgG, and with human Fc fragments (Table 1).

DISCUSSION

Our results clearly demonstrate that pooled IgG preparations can improve experimental autoimmune haemolytic anaemia induced in mice with an anti-erythrocyte MoAb. This therapeutic activity appears to be related to macrophage blockage, since no effect is observed when the anaemia is induced by an anti-erythrocyte MoAb that does not trigger phagocytosis. This may explain why IgG treatment failed to reduce the spontaneous anaemia of NZB mice, which have autoantibodies capable of destroying erythrocytes by various mechanisms (data not shown), or of C3H mice that develop autoimmune haemolysis after infection with lymphocytic choriomeningitis virus [35–37].

Our results in an experimental mouse model fit well with observations reported in patients with haematologic autoimmune diseases. *In vivo* IgG inhibits the clearance of antibody-coated erythrocytes by reacting with macrophage Fc receptors, and *in vitro* decreases rosette formation around macrophages [38,39]. Such an effect on macrophage function has been correlated with an increase in platelet count in patients with autoimmune thrombocytopenia. Treatment with either Fc fragments [31], anti-Rh antibody [40] or a MoAb reacting with macrophage Fc receptor [41] produced the same effect. It has therefore been proposed that this rise in platelet count was due to the blockage of Fc receptors by pooled IgG [42]. Such a mechanism might also be involved in the successful treatment of some patients with autoimmune haemolytic anaemia [43,44]. The variability of treatment efficiency in these patients may suggest that other mechanisms are also operating, such as the action of anti-idiotypic antibodies present in the therapeutic IgG preparations. However, it may also be related to the presence of autoantibodies with different pathogenic mechanisms, such as those used in our experimental model. The strong similarity between *in vivo* IgG efficacy in mice that received anti-erythrocyte MoAb and the results of *in vitro* inhibition of erythrophagocytosis suggest therefore that it could be possible to develop a simple *in vitro* therapeutic assay to predict the effect of IgG treatment in patients with haemolytic anaemia. Such an assay could allow for the more rational use of pooled IgG infusion, which is both expensive and has been associated with viral infections such as hepatitis C, by identifying those patients who could be expected to benefit from such treatment.

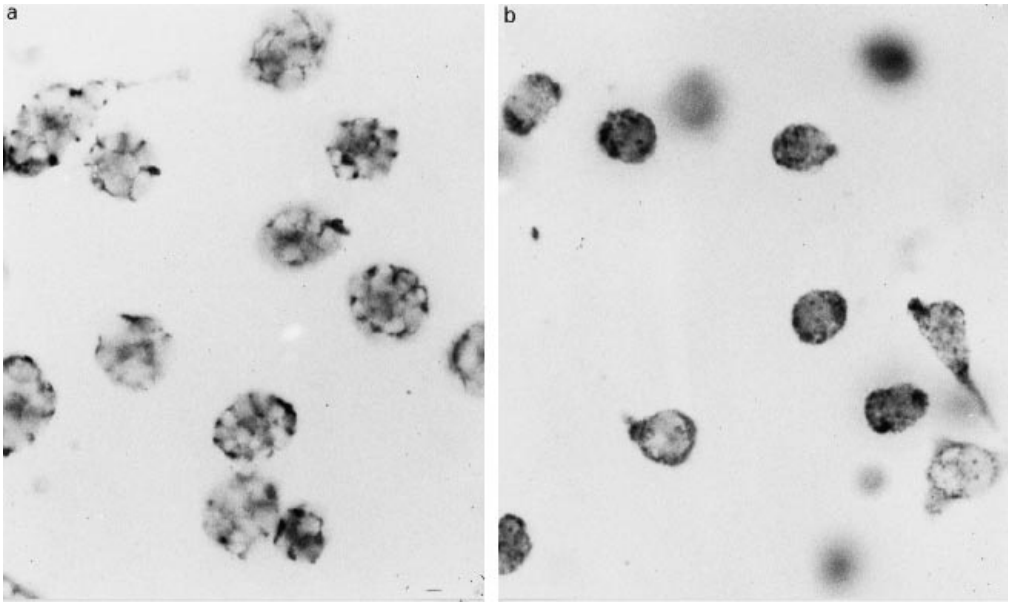

Fig. 3. Inhibition of *in vitro* erythrophagocytosis with total IgG. Peritoneal macrophages from BALB/c mice were incubated with erythrocytes sensitized with 34-3C MoAb, in the presence of buffer alone (a), or 200 μg total human IgG (b). All macrophages shown in (a) have phagocytized at least five erythrocytes, whereas macrophages shown in (b) have not.

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