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Haemolytic disease in mice induced by transplantation of hybridoma cells secreting monoclonal anti-erythrocyte autoantibodies

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It is presently unknown if autoimmune responses are similar to other immune responses, which are polyclonal. An autoimmune response could be invoked by many different monoclonal antibodies, and disease caused by their combined effects. The cell hybridization technique allows analysis of lymphocyte clones and their monoclonal products, and hybridomas which secrete defined monoclonal autoantibodies can be used to determine the role of such antibodies in autoimmunity. Autoimmune haemolytic disease (AIHD) results from a pathogenic anti-erythrocyte response associated with the binding, *in vivo*, of antibodies directed against antigens present on the red cell surface. The disease is characterized by a positive direct Coombs' antiglobulin test, and anaemia.

This paper describes the production and characterization of lymphocyte hybridomas secreting monoclonal autoantibodies against red blood cells (RBC). When transplanted into syngeneic or allogeneic nude mice such hybridoma cells induce AIHD in the recipients. One cell line, KIE3, induced a monoclonal autoimmune haemolytic disease that was fatal within 2 weeks.

Hybridoma cell lines were established according to the fusion technique of Galfré, Howe, Milstein, Butcher & Howard (1976). Lines were cloned in semi-solid or fluid phase media by limiting dilution and their monoclonal products were identified and assayed in a microhaemagglutination assay (Table 1). Three hybridomas and the myeloma parent line P3.X63.Ag8/NS-1 (NS-1) of BALB/c origin, were used in experiments described here. Two hybridomas were derived from the fusion of NZB spleen cells with NS-1 cells and secreted respectively, a monoclonal IgM autoantibody (KIE3) and an IgG2a autoantibody (KIE4). A third line was derived from CBA spleen cells and secreted an IgG1 autoantibody (KIE1). The three monoclonal antibodies agglutinated both mouse and rat RBC, their titres always being higher with rat RBC. The KIE3 antibody had the highest relative reactivity with mouse RBC when the agglutinating activity of each antibody against rat RBC was compared with the normalized activity against mouse RBC (Table 1).

Mice implanted with these hybridomas developed haemolytic disease, the severity of which varied with the particular hybridoma transplanted and with the histocompatibility relationship between the hybridoma and the recipient. The results in Table 2

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Table 1. Hybridomas secreting monoclonal anti-RBC autoantibodies

Cell line	KIE1	KIE3	KIE4
Strain of origin	CBA	NZB	NZB
Class of antibody secreted	IgG1	IgM	IgG2a
Haemagglutinating activity Log_2 (rat RBC titre/mouse RBC titre)	11	6	17

The hybridomas were produced by the fusion of NS-1 cells with spleen cells from mice given a regime of intraperitoneal injections with rat erythrocytes (Playfair & Marshall-Clarke, 1973), and the spleen cells were fused 3 days after the last injection. The monoclonal antibodies secreted by the hybrid cells were reactive against rat and mouse RBC. The class of antibody secreted was determined by double-diffusion in gel using commercial antisera (Meloy).

Indirect haemagglutination test. Twenty-five microlitres of doubling dilutions of hybridoma culture supernatants were incubated for 1 hr with 2 μl of a 5% v/v suspension of RBC [washed twice in 0·15 M NaCl then twice in RPMI 1640 tissue culture medium supplemented with 5% foetal calf serum (FCS), Flow Laboratories, Ltd]. Two microlitres of diluted rabbit anti-mouse Ig antiserum (Nordic) were added for a further period of 1 hr. Titres are given as the highest dilution of supernatant causing haemagglutination. The assay was conducted at 37° and all reagents were diluted in RPMI 1640 containing 5% FCS.

summarize the disease process in mice carrying syngeneic hybridoma cells. The most extreme disease was induced by the IgM autoantibody secreted by hybridoma KIE3: this was rapidly fatal in all the (BALB/c × NZB)F_1 mice. AIHD of a similar severity also occurred in CBA *nu/nu* mice. A positive antiglobulin test, reduced haemoglobin level and reticulocytosis diagnostic of AIHD were prominent features of the haemolytic process in all KIE3-implanted mice. There was a marked splenomegaly, the organ showing severe congestion of the red pulp and extensive erythrophagocytosis. Haemosiderin was prominent in the Kupffer cells of the liver, indicative of phagocytosis and destruction of RBC. No significant changes in thymic weight or morphology were seen in mice implanted with KIE3 dying with severe haemolytic anaemia. By contrast, animals implanted with KIE4 died later, showed tumour development and atrophy of the thymus. There was no evidence of tumour in mice with advanced haemolytic disease induced by KIE3 in any of the tissue sections examined, but tumour cells were seen in smears of peripheral blood. There was also marked polychromatophilia, autoagglutination of RBC, erythrophagocytosis by mononuclear cells, and normoblastsis, all of which are indicative of a severe autoimmune haemolytic process.

In contrast with the IgM KIE3 antibody, the IgG2a (KIE4) and IgG1 (KIE1) monoclonal autoantibodies caused changes associated with a haemolytic process with a later onset and slow progression which occurred in 75% of the KIE4-implanted mice, and in 20% of the KIE1-implanted mice. Tumour developed predominantly in the abdominal organs of the mice carrying the KIE4 or KIE1 hybridomas, sometimes to such an extent as to cause internal bleeding. When the evidence from post-mortem examination and histological studies was compared with the haematological profile it was clear that a haemolytic process was only found in the tumour-bearing mice. However, the haematological changes were less pronounced in these mice than in those implanted with KIE3, and the mice did not have a positive antiglobulin test, but there was clear evidence of reticulocytosis and haemoglobinaemia. Splenomegaly was probably due to tumour growth since spleen sections displayed very little RBC accumulation and no deposits of haemosiderin.

In control experiments, NS-1 myeloma cells (which do not secrete anti-RBC autoantibodies) did not grow in (CBA × BALB/c)F_1 mice, and only formed solid tumour in five out of eight (BALB/c × NZB)F_1 mice. In all the mice a leucocytosis was recorded initially. The (CBA × BALB/c)F_1 mice were healthy when the blood profile was examined 89 days after implantation and numbers of the WBC, percentage of reticulocytes and haemoglobin levels were in the normal range. No tumour had developed by 184 days. In the (BALB/c × NZB)F_1 tumour-bearing mice the leucocytosis persisted and there was also a mild reticulocytosis. Haemoglobin levels were within the normal range, and there was no increase in the antiglobulin titre.

KIE1 and KIE4 have effects not seen with NS-1. The interpretation of the data must accommodate the fact that the tumours themselves cause haematological changes irrespective of their property of antibody secretion. However, the data indicate that the KIE1 and KIE4 antibodies do have a pathogenic effect in causing a haemoglobinaemia which is not associated with NS-1 tumour cells. Thus, in comparing the three monoclonal autoantibodies, it can be seen (i) that they differ in their ability to induce the changes classically associated with an autoimmune haemolytic process,

Table 2. Transplantation of hybridomas in histocompatible recipients

Host	Cell line	Time killed (days)	Spleen (mg gm^{-1})	Mes. node (mg gm^{-1})	Thymus (mg gm^{-1})	WBC ($\times 10^{-6}$ dl^{-1})	Reticulocytes (% RBC)	Hb (gm dl^{-1})	Antiglobulin titre (Log_2)
(BALB/c × NZB) F_1	Nil ($n=6$)		3·8 ±0·8	2·0 ±0·5	1·0 ±0·3	4·7 ±2·1	4·5 ±3·0	14·9 ±1·4	—
	KIE3	9	16·1	2·5	1·4	12·9	42	4·1	6
	KIE3	9	19·7	2·2	1·0	15·3	67	7·2	6
	KIE3	9	12·8	2·3	0·9	21·2	41	8·8	3
	KIE3	8	12·2	1·7	0·5	11·8	41	7·1	6
	KIE4	22	9·3	1·6	1·6	6·0	29	4·2	—
	KIE4	22	9·8	1·4	0·9	8·0	34	2·6	—
	KIE4	37	3·3	>5·0	0·3	5·2	21	8·0	1
	KIE4	46	4·4	>5·0	0·5	5·0	11	14·5	—
	KIE4	99	8·6	>5·0	ND*	6·3	30	ND	—
	NS-1	46	4·8	>5·0	0·7	ND	15	13·1	—
	NS-1	31	4·2	>5·0	1·1	2·4	12	16·1	—
(CBA × BALB/c)F_1	Nil ($n=6$)		2·9 ±0·5	1·6 ±0·4	1·2 ±0·4	4·7 ±0·7	3·5 ±2·1	14·5 ±1·8	—
	KIE1	59	23·2	>5·0	ND	2·9	6·0	8·8	—

* ND, not done.

Hybridoma cells, washed once in isotonic saline and suspended at $5–10 \times 10^7$ cells ml^{-1} in saline were transplanted intraperitoneally (0·5 ml) into histocompatible mice. The mice were observed daily and killed at the times indicated. Blood samples were obtained by cardiac puncture. White blood cell (WBC) concentration was determined by haemocytometer counting, and reticulocyte concentration was found by staining blood smears with cresol-blue using Merret tubes (Mercia-Brocades Ltd). Haemoglobin (Hb) concentrations were determined by the cyanmethaemoglobin method (Dacie & Lewis, 1975). The antiglobulin titre was estimated by adding 2 μl of a 5% v/v suspension of washed RBC to 25 μl of doubling dilutions of rabbit anti-mouse Ig antiserum under conditions similar to those described in Table 1. Titres are expressed as the highest dilution of antiglobulin reagent causing haemagglutination after incubation for 1 hr at 37°.

All animals represented in the table were killed because they showed clinical symptoms of AIHD or tumour. Organ weights are given as mg per unit body weight. Some animals died from disease, in which case it was not possible to determine accurate haematological measurements or organ weights. Control values from normal mice are expressed as mean ± SD.

and (ii) that they cause symptoms such as haemoglobinaemia which can occur in the absence of detectable membrane-bound autoantibody and histological changes in the spleen.

The acute haemolytic disease occurring in KIE3-implanted mice was quite distinctive. The evidence from post-mortem examination and histological studies points to a severe autoimmune haemolytic process occurring in the absence of any significantly disseminating solid tumour. Thus AIHD was induced by the monoclonal autoantibody secreted by the transplanted KIE3 hybridoma cells. This hybridoma is semi-allogeneic in NZB and CBA hosts and did not grow progressively in them. Such mice, however, developed a reticulocytosis, suggestive of a haemolytic process, but their blood profile had returned to normal by 18 days and the mice remained healthy for at least 106 days.

The haemolytic diseases caused by the monoclonal autoantibodies secreted by the hybridomas are not identical to the spontaneous AIHD of NZB mice, which appears to depend upon autoantibody directed against the X antigen (Bielschowsky, Helyer & Howie, 1959; Helyer & Howie, 1963; Linder & Edgington, 1972) and is usually thought to be of the IgG class (Linder & Edgington, 1971). The monoclonal autoantibodies in this study have specificities different from those found in the spontaneous disease: whereas the hybridoma antibodies react with both rat and mouse RBC, it has been shown that the spontaneous autoantibody reacts only with mouse RBC (Cooke, Hutchings & Nayak, 1980). Furthermore, in haemolytic disease induced in mice immunized with rat RBC, the anaemia appears to correlate with the presence of IgG2b antibodies (Cox & Lin Ying Koh, 1977; Milch & Gershwin, 1979).

The present experiments show that haemolytic disease can be caused by antibodies of isotypes not normally detected in the spontaneous or rat erythrocyte-induced disease states. The hybridoma technique has been exploited in order to isolate cloned lymphocytes and their monoclonal products in quantity sufficient for extensive analysis. In the process of using this strategy it has been possible to circumvent the restrictions inherent in studying autoantibodies eluted from RBC of antiglobulin positive mice where it is likely that antibodies with a powerful destructive potential for RBC would promote rapid removal from the circulation of the cells carrying them, and thus be undetectable by a direct antiglobulin test or by elution.

The monoclonal antibody that caused the most severe haemolytic disease was derived from an NZB mouse. Its powerful pathogenicity may indicate that it is one of the antibodies responsible for the spontaneous haemolytic disease of such mice and that further, there is a genetic predisposition of these animals, and not of animals of other strains, to produce such antibodies.

It is not known how many different antibody clonotypes contribute to the spontaneous AIHD of NZB mice. If there are many, then immunological manipulation of such a process for therapeutic purpose would be greatly complicated. Despite this stricture, the type of approach used here could be of great value in elucidating the importance of isotypy and idiotypy for the pathogenic effects of such monoclonal autoantibodies. It will therefore be important to investigate further the range of such autoantibodies in mouse strains susceptible to or resistant to the development of spontaneous haemolytic anaemia, as well as to raise antisera, particularly against idiotypic specificities, to assess their role in the modulation of haemolytic disease caused by such antibodies.

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