

AD-A210 038

REPORT DOCUMENTATION PAGE

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1a. SECURITY CLASSIFICATION AUTHORITY Unclassified		1b. RESTRICTIVE MARKINGS NO FILE COPY	
2a. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution is unlimited	
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 89-31		7a. NAME OF MONITORING ORGANIZATION Naval Medical Command	
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research		6b. OFFICE SYMBOL (If applicable)	
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Naval Medical Research and Development Command		8b. OFFICE SYMBOL (If applicable)	
8c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
10. SOURCE OF FUNDING NUMBERS		11. TITLE (Include Security Classification) Production of human monoclonal rheumatoid factor secreting hybridomas derived from rheumatoid synovial cells	
12. PERSONAL AUTHOR(S) Robbins DL, Kenny TP, Larrick JW, Wistar R		13. TIME COVERED FROM TO	
13a. TYPE OF REPORT journal article		14. DATE OF REPORT (Year, Month, Day) 1989	
15. SUPPLEMENTARY NOTATION Reprinted from: Medical Science Research 1989 Vol.17 pp. 157-159		16. PAGE COUNT 3	
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD GROUP SUB-GROUP			
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
DTIC ELECTE JUL 06 1989 S D			
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division		22b. TELEPHONE (Include Area Code) 202-295-2188	
		22c. OFFICE SYMBOL ISD/ADMIN/NMRI	

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

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Production of human monoclonal rheumatoid factor secreting hybridomas derived from rheumatoid synovial cells

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Although the cellular arm of the immune system is undoubtedly important in the pathogenesis of rheumatoid arthritis (RA), RA is an autoimmune extravascular immune complex disease involving synovium and interstitial tissues. The major autoantibody present is rheumatoid factor (RF), a polyclonal autoantibody directed to the Fc portion of IgG. Several observations point to RF-IgG complexes and their subsequent activation of complement as being important in the pathogenesis of RA [1, 2]. RF are heterogeneous in many qualitative characteristics including complement activating properties, specificity and avidity. For example, exhaustive studies attempting to demonstrate unique antigenic determinants on IgG Fc towards which serum RF is directed have been inconclusive [1], although many RA serum RF react with an antigen in the Fc that binds staphylococcal protein A [3]. RF synthesis is the major function of synovial tissue plasma cells in RA and intra-synovial immune complex formation is a predominant feature of RA synovitis [4]. Synovitis is central to the immunopathologic events in RA, thus it is likely that RF made there has greater pathogenetic importance than serum RF.

Characterizing the molecular genetic basis of RF in RA could provide exciting new understanding of important genetic and triggering factors in RA. However, this has not been possible because of the polyclonality of RF in RA. Hybridoma technology should overcome these difficulties and allow more precise characterization of these polyclonal RF, but reports to date using peripheral blood lymphocytes (PBL) have described only limited success [5]. We herein describe successful production of human monoclonal IgM RF (mRF) secreting hybridomas derived from rheumatoid synovial cells (RSC).

Materials and methods: Synovial tissue was obtained incidentally to clinically indicated surgical procedures on RA patients as previously described [6]. Briefly, RSC were washed during tissue processing to remove absorbed serum RF. Specimens were collected in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution containing heparin and antibiotics and were processed immediately. The tissue was minced and dissociated by treatment with collagenase and DNase, followed by filtration through nylon mesh. The eluted cell suspension was centrifuged and the cells were resuspended in tissue culture medium supplemented with 10% fetal calf serum (RPMI-FCS). The cells were washed three times and resuspended in RPMI-FCS and antibiotics. Cells were counted in a hemocytometer and viability was assessed by Trypan blue dye exclusion.

With slight modification, RSCs were fused as described previously [7] to generate hybridomas secreting human monoclonal IgM RF. RSC and mouse/human cell line F3B6 cells were washed separately in Hanks balanced salt solution without calcium and with 2 mM magnesium (HBSS-/+). At a 1:1 ratio the cells were mixed and 2×10^7 cells/well were added to peanut agglutinin (PNA) precoated Falcon 6-well plates (prior to fusion the wells were incubated with 5 $\mu\text{g}/\text{ml}$ of PNA at 37°C). The plates were centrifuged at $500 \times g$ for

6 min at room temperature. The supernatant was aspirated off the monolayer and 2 ml of warmed (37°C) 40% polyethyleneglycol (PEG) fusion mixture (8 g PEG 4000, 2 ml dimethylsulfoxide (DMSO) with HBSS-/+ to a final volume of 20 ml) was added down the side of the wells. After 1 min, 4 ml of warm HBSS-/+ with 5% DMSO (FDM) was slowly added over the next 2 min; 4 ml of FDM was then added over 1 min. The wells were aspirated and 2 ml of HBSS-/+ was added and the plate was centrifuged at $400 \times g$ for 5 min. The fluid was aspirated and another 2 ml of HBSS-/+ was added and the plate was respun for 5 min. The HBSS-/+ was aspirated and 3 ml of growth medium (GM) was added to the wells. The plate was incubated overnight at 37°C in 5% CO_2 . The following day the cells were diluted with growth medium containing 100 μM hypoxanthine and 2 $\mu\text{g}/\text{ml}$ azaserine (HAGM). The cells were gently resuspended and seeded into 96-well flat bottom plates, 1×10^4 cells/well. The cells were fed with HAGM every 3rd day. Hybrids were usually ready to assay by day 14 after fusion.

The growth medium used was Iscoves Modified Dulbecco's Medium supplemented with prescreened 10% fetal calf serum (FCS) (heat inactivated), 5.0×10^{-3} IU/ml bovine insulin, 5.0 $\mu\text{g}/\text{ml}$ human transferrin, 5 ng/ml of sodium selenite and 5 $\mu\text{g}/\text{ml}$ human low density lipoprotein (LDL) and 50 IU/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin.

Cloning of suspected IgM RF hybrids was done as follows. Cells from IgM RF positive wells were counted and diluted to 300 cells/ml with growth medium supplemented with 15% FCS. From this stock, 1:10 dilutions were made giving two other concentrations, 30 cells/ml and 3 cells/ml. 0.1 ml of the stocks were dispensed into 96-well U-bottom plates. Plates were fed every 4 days and assayed on day 12. The positive wells of the last dilution giving less than 36% (Poisson Formula) positive wells were presumed to be monoclonal. Several of these wells were then recloned until 100% of the wells with growing cells secreted monoclonal IgM RF.

Cells at log phase in their growth cycle were centrifuged at $200 \times g$, resuspended in FCS with 5% DMSO and aliquoted into freeze vials (Costar). The vials were placed in a -90°C freezer for 12 h and then immersed in liquid nitrogen.

The enzyme immunoassay (ELISA) for RF IgG and IgG subclass specificity was performed as described previously [8]. Briefly, plastic microtiter plates were incubated with 60 $\mu\text{g}/\text{ml}$ IgG or IgG subclasses diluted in PBS. The wells were washed and then quenched. The buffer was removed and the various samples and standards containing IgM RF were added. After incubation at room temperature, the wells were washed. Goat anti-human IgM labeled with horse radish peroxidase was then added to the wells. After incubation, the wells were washed. ABTS diluted in citrate buffer was freshly prepared for each assay. 3% H_2O_2 was added to ABTS immediately before addition to the wells. The color was allowed to develop and then stopped. Absorption at 405 nm was measured. Background binding derived from wells that were not precoated with IgG was subtracted.

Polyclonal human IgG was prepared by purifying human Cohn Fraction II further by ion exchange chromatography on DEAE cellulose as described earlier [6]. To prepare monomeric IgG, the respective IgG preparations were ultracentrifuged at $100\,000 \times g$ for 60 min at 4°C . After this the upper one third of the solution was collected and the $\text{OD}_{280\text{nm}}$ of an aliquot was read to determine protein concentration. Myeloma IgG subclass proteins were isolated using ion exchange chromatography followed by gel permeation chromatography where indicated [6]. An ELISA inhibition assay was used to assess the amount of contamination of purified human myeloma proteins with other subclasses [6]. The sensitivity of this assay is in the range of 0.5 to $2\ \mu\text{g/ml}$ for the homologous antigen. All of the subclass proteins used in our studies were 98 to 99% pure as determined by this method. All IgG preparations used were negative for RF activity when examined by ELISA [6].

Results: The results of fusions done on the RSC of four seropositive RA patients are outlined in Table 1. As is seen, the frequency of positive clones for RSC IgM monoclonal RF secretion varied between 2.5 and 3.8%. As a control, synovial lymphocytes obtained from patients with other types of arthritis who were seronegative were also tested. As can be seen in Table 1 there were no IgM RF secreting hybrids produced.

Table 1: Frequency of IgM RF secreting hybrids from rheumatoid synovial cells in seropositive rheumatoid arthritis patients and from synovial cells in arthritis patients seronegative for rheumatoid factor.

Patients	No. of hybrids*	IgM RF Hybrids	%
Seropositive			
VH	450	16	3.6
MK	320	12	3.8
LC	240	7	2.9
VJ	120	3	2.5
Seronegative			
FO	280	0	0
DO	87	0	0
SE	202	0	0

*Number of microtiter wells in which cell colonies were observed.

The results of primary screening by ELISA for RSC RF IgG subclass specificity from patient MK in Table 1 are shown in Table 2. As is seen, each RSC IgM mRF showed its own individual IgG subclass binding profile. Because these data were obtained early in the subcloning process, although unlikely, the possibility existed of more than one clone per well.

Table 2: Rheumatoid synovial cell mRF subclass specificity profiles (ELISA) from patient MK in Table 1*

Well	IgG1	IgG2	IgG3	IgG4
AD7	+++	++	-	+
AG4	+	+	+	+-
AG6	+	+	-	-
CB6	++	++	+	-
CD4	++++	++	-	++
CD9	++++	++++	-	++++
CG9	+	+	-	+
CE3	+	+	+	+
CC9	+	-	-	-
CF11	+	+-	-	+-
CB9	+	+-	-	+-
DG5	++	+	-	++

*Screening was done with hybridoma supernates

When the most stable clone, AD7, which showed binding to IgG1, IgG2 and IgG4, but no binding to IgG3 was subcloned an additional three times, the percentage of RSC IgM mRF secreting hybrids increased from 80 to 100%.

The anti-IgG specificity of AD7 was confirmed by examining its binding to a panel of antigens using a direct binding ELISA. As is seen in Figure 1, IgM RF AD7 bound only to human IgG out of all of the antigens employed.

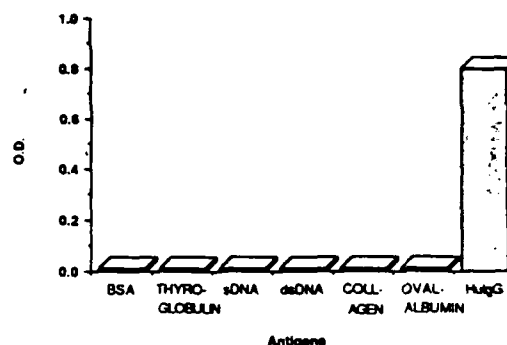


Figure 1: Binding of RSC derived IgM mRF AD7 to a panel of antigens and to human IgG. AD7 bound only to human IgG demonstrating its anti-IgG specificity.

We were then interested to see whether AD7 RF had preferential or cross-species reactivity. AD7 RF reacted with human and rabbit IgG when examined by direct binding ELISA (Figure 2).



Figure 2: Reactivity of AD7 mRF with IgG from multiple species measured by direct binding ELISA (IgG as precoat antigen).

We then characterized AD7 RF to be certain that it was IgM isotype, monoclonal, and expressed no mouse gene products. This was done in a direct binding ELISA using AD7 in the solid phase and appropriate antisera to human IgM and human IgG heavy chains, kappa and lambda light chains, and antisera to whole mouse Ig including light chains. AD7 RF was a human IgM k monoclonal antibody without evidence of expressed mouse gene product (Figure 3).

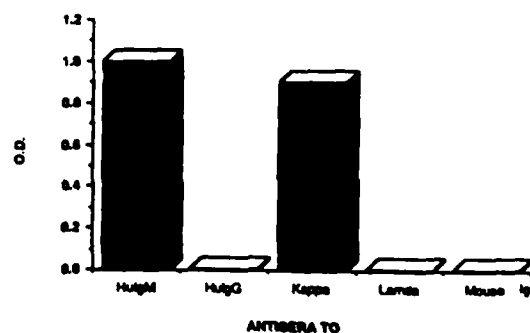


Figure 3: Characterization of AD7 RF by direct binding ELISA. AD7 was used as precoat antigen against antisera as indicated.

Discussion: The ability to study the molecular genetic basis of RF in RA has been precluded by its polyclonality. Our RSC hybridoma derived human monoclonal IgM RF should overcome many of those difficulties and offer advantages over previously described methods. First, they are derived from RSC rather than from PBL and are more likely to represent pathogenetic RF. Second, our RSC hybridomas are not stimulated with EBV or other mitogens. This is possible because RF in RA is preferentially synthesized in RSC compared with PBL [6, 8]. Although studies using hybridoma technology may be accompanied by some uncertainty as to the genealogy of the immortalized B cells, we believe that our hybrids should provide optimal opportunity to study V_H genes and gene products that have been encoded for and triggered *in vivo* rather than as an artifact of stimulation or hybridoma evolution [9, 10]. Moreover, we have found no evidence for expressed mouse gene products. Third, our RSC hybridoma mRF do not react with antigens other than IgG in contrast to those reported by others derived from human PBL [5] and each of our RSC mRF has its own distinctive subclass specificity profile.

In conclusion, better understanding of the molecular genetic basis of RSC RF may allow us to determine the role of qualitative characteristics of RF such as isotype, affinity, and fine specificity in pathogenicity. Moreover, a precise analysis of the molecular genetic basis of RF and their antigenic and idiotypic specificities may tell us how they are elicited and how these molecules escape normal regulatory

networks [9]. The RSC hybridoma described here should allow us and others to define better the origin and heterogeneity of RF in RA. Further, observations such as these may also be applicable to autoantibody production in other autoimmune diseases.

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The authors thank Nikki Rojo for her excellent secretarial assistance and Kim Robbins for her excellent technical assistance.

Supported in part by NIH grant AM 32287.

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Paper received: 10th January, 1989

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 JAN 1989
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