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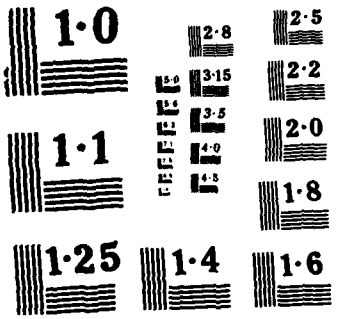

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December 19, 1985

Eli D. Schmell
Program Manager
Molecular Biology Program, Code 441MB
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217

Dear Dr. Schmell:

Enclosed please find the final report for Contract N00014-82-K-0221, "Macrophage Structure and Function", for the period of 2/1/82 - 1/31/85.

Sincerely,

Thomas August

TA/lp

Extensive progress has been made in the identification and characterization of several proteins active in the human immune response. These results are summarized as follows:

a. PREPARATION OF MONOCLONAL ANTIBODIES;

Balb/c mice were immunized with the adherent fraction of human peripheral blood mononuclear cells which included monocytes and dendritic cells. Spleen cells from the immunized mice were used to derive antibody-secreting hybridoma cell lines according to standard procedures (Hughes and August, 1981). The monoclonal antibodies produced were screened in three ways:

i. Analysis of the specificity of antibody binding to cells by immunoperoxidase localization of antibody binding on cryostat sections of human tonsil.
ii. Immunoprecipitation of specific proteins.
iii. Effect on in vitro T-cell response:
   Phytohemagglutinin (PHA) stimulation
   Mixed lymphocyte reaction (MLR)
   Soluble antigen induced proliferation
   Cytotoxic T-lymphocyte mediated lympholysis (CTL)
   Natural killer cell cytolysis (NK)

These cell fusions and the screening process were highly successful, yielding over 80 monoclonal antibodies that are now being characterized.

b. IDENTIFICATION OF CELL PROTEINS;

Cont'd pg. 3

Several of the proteins targeted by these antibodies have been identified as previously described molecules by cell specificity, molecular weight, and function (Table 1). These antibodies and proteins have significant value to this project and we currently are working with the human lymphocyte function antigen (HLFA), Mac-1, and the HLA Class II proteins, as described below.

The largest number of immunoprecipitated proteins are, to our knowledge, unidentified (Table 2). We currently are characterizing 2 of these proteins, (Mon.1 and Mon.2) that appear to be involved in novel immune response functions, as described below. Many of the other proteins show properties of considerable interest and may be studied in the future, as time and resources permit, particularly Mon.3, that blocks T cell proliferation.

TABLE 1

MONOCLONAL ANTIBODIES REACTING WITH PREVIOUSLY IDENTIFIED ANTIGENS OF HUMAN BLOOD CELLS

Number of Antibodies	Polypeptides Precipitated (kDa)	Antigen Identified	Antigen Function
5	33, 28	HLA Class II	Antigen recognition
1	45	HLA Class I	Antigen recognition
3	175, 95	Lymphocyte function antigen	Cell adhesion
3	165, 95	Mac-1	Complement receptor-3
2	200	Lymphocyte common antigen	---
1	190, 93	Transferrin receptor	Iron transport

TABLE 2

PREVIOUSLY UNIDENTIFIED PROTEINS OF HUMAN BLOOD CELLS REACTING WITH MONOCLONAL ANTIBODIES

Number of Antibodies	Polypeptides Precipitated (kDa)	Antigen Identified	Antigen Function
1	50	Mon.1 (H2A9)	Induction of MLR
1	85	Mon.2 (H4C4)	Induction of MLR
1	200	Mon.3 (H6A7)	T cell proliferation
7	64	(plas memb, serum, MLR sup)	---
2	50,55,60	(serum antigen)	---
6	120	(macrophage)	---
2	70,50,20	(monocytes)	---
1	80,50,24,20	(monocytes)	---
2	130	(endothelial cells)	---
2	23	(adherent cells)	---
1	40	---	---
1	50	---	---
1	32	---	---
1	111	---	---
1	170,85	---	---
1	50	---	---

c. CHARACTERIZATION OF THE HUMAN LYMPHOCYTE FUNCTION ANTIGEN (HLFA):

Five monoclonal antibodies, all of the IgG1,k isotype, are being used to characterize the HLFA. Immunoprecipitation of proteins from extracts of T cells or monocytes show that a single β-subunit of Mr=95,000 is shared by 3 different α-subunits of Mr=175,000, Mr=165,000 and Mr=150,000 (Table 3). Three of the antibodies (Mon.4, Mon.5, MHM.23) are specific for the β-subunit; another (MHM.24), with HLFA-α; and the fifth (Mon.6), with HMac-1 α.

TABLE 3

ANTIBODIES DIRECTED AT THE

HUMAN LYMPHOCYTE FUNCTION ANTIGEN

Monoclonal Antibody	Polypeptides precipitated (Mr X 10^{-3})				Specificity
	T cells		Monocytes		
	α	β	α	β	
Mon.4	175,	95	175, 165, 150,	95	β
Mon.5	175,	95	175, 165, 150,	95	β
MHM.23	175,	95	175, 165, 150,	95	β
MHM.24	175,	95	175,	95	HLFA-α
Mon.6	--		165,	95	HMac-1-α

Our major interest is the biological affect of the antibodies:

Mixed lymphocyte reaction: The β-subunit specific antibodies (Mon.4, Mon.5, MHM.23) as well as the HLFA α-subunit-specific antibody (MHM.24) markedly inhibit the human mixed lymphocyte reaction. These antibodies also completely block the generation of cytotoxic T cells in the mixed lymphocyte reaction. The anti-HMac-1 α antibody Mon.6 shows no effect on the mixed lymphocyte reaction.

Phytohemagglutinin stimulation: The three β-subunit-specific antibodies and the HLFA α-subunit-specific antibody completely inhibit the response of lymphocytes to a suboptimal dose (0.1 μg/ml) of PHA. The lymphocyte response to the mitogen is not affected by the HMac-1 α-specific antibody (Mon.6).

Cytotoxic T lymphocyte mediated lymphosis: Similarly, the three β-subunit-specific antibodies and the HLFA α-subunit-specific antibody completely inhibit lysis of target cells by cytotoxic T cells.

Natural killer cell lympholysis: The three β-subunit-specific antibodies (Mon.4, Mon.5, MHM.23) inhibit NK cell actolysis by 80 percent whereas the HLFA α (MHM.24) and HMac-1 α (Mon.6) specific antibodies have no effect.

d. THE IN VITRO FORMATION OF MULTINUCLEATED GIANT CELLS INDUCED FROM MONOCYTES BY MONOCLONAL ANTI-HLA-DR: Multinucleated giant cells (MGC) arise in granulomas caused by bacterial or viral infections or by physical or chemical agents, and are also encountered in granulomatous diseases of unknown etiology such as sarcoidosis, rheumatoid arthritis, and Crohn's disease. We recently have made the novel observation that multinucleated giant cells can be induced in vitro by incubating human peripheral blood monocytes with anti-HLA-DR monoclonal antibodies.

Cont'd 9.6

Our studies began in the initial screening of the monoclonal antibodies described above, when it was observed that certain antibodies caused the formation of large aggregates of cells. It was found that all of the positive antibodies were anti-HLA-DR. The possible association was obvious since macrophages present antigen in association with products of class II major histocompatibility complex (MHC) genes to antigen-specific T cells.

Two hours after adding HLA-DR specific MAb to human monocyte cultures large aggregates of adherent and non-adherent cells are observed (Figure 1a). After 24 hours of culture with HLA-DR specific MAb almost all of the cells gather into very large clusters consisting of several cells (Figure 1b). In control cultures a uniform monolayer of adherent cells is observed at this time. After an additional 24 hours of culture many of the large clusters differentiate into MGC of the foreign-body type (FB-MGC) (Figure 1c). The predominant cell type at day 2 is a very large multinucleated (10-60 nuclei) polygonal epitheloid cell with 2 to 4 cytoplasmic extensions. Six to 12 hours later these cytoplasmic extensions spread and fuse, resulting in very large FB-MGC. After 72 hours of culture with the HLA-DR specific MAb, 80-90 percent of the nuclei are seen in MGC (Fig. 1d). The MGC continue to fuse such that by 4 to 5 days of culture, polykaryons with greater than 100 nuclei are observed. In monocyte cultures without HLA-DR specific MAb, greater than 90 percent of the cells are present in the non-adherent population after 3 days of culture. Monocytes treated with anti-HLA-DR MAb do not incorporate ^{3}H-thymidine, confirming that MAb-induced MGC are formed by fusion, not by nuclear division.

FIG. 1

Multinucleated Giant Cells Induced

By Anti-HLA-DR Monoclonal Antibody

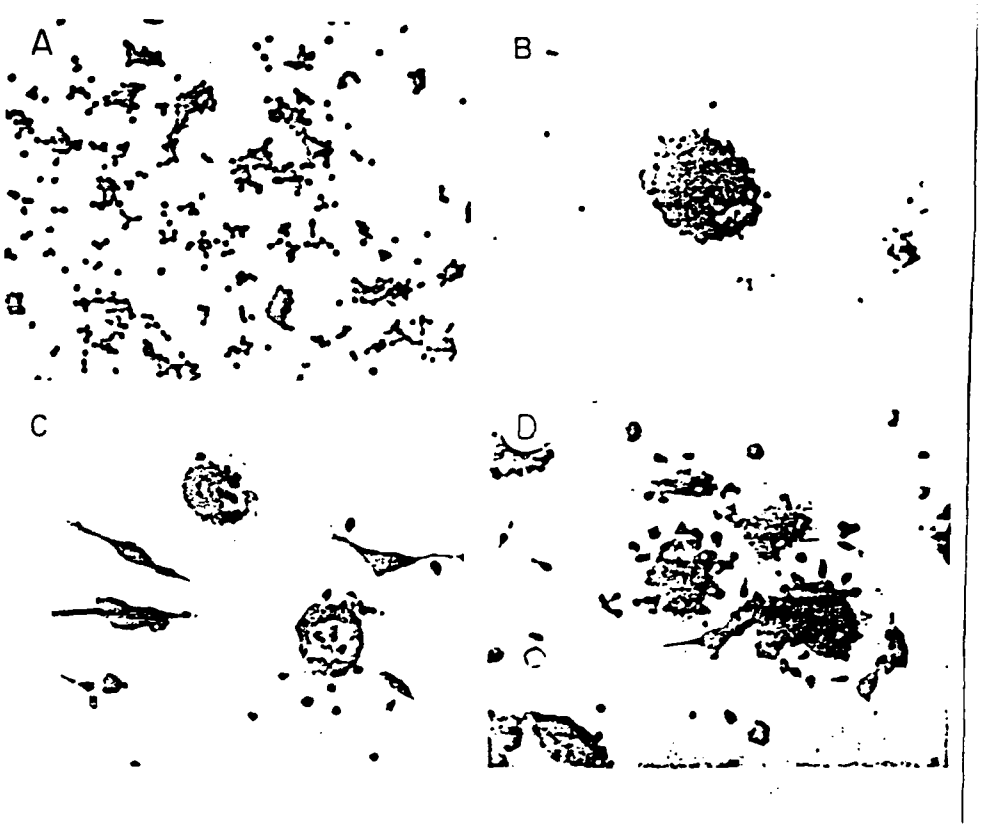

Figure 1:

Time course of monoclonal antibody induced multinucleated giant cells. Peripheral blood mononuclear cells (PBMC) were isolated from healthy adult donors by Ficoll-Hypaque density centrifugation. Monocytes were isolated by centrifugation of PBMC on Percoll density gradients. Five X 10^6 monocytes in 5 ml of RPMI-1640 (Biofluids) supplemental with 10 percent heat-inactivated fetal bovine serum (FBS) (Hyclone) were cultured in 28 cm^2 petri dishes (Falcon) at 37°C in a humidified atmosphere of 5 percent CO_2. Monoclonal anti-HLA-DR antibody (immune ascites) was added for a final dilution of 1:5000. At the indicated times, the non-adherent cells were washed away and the adherent cells were fixed with absolute methanol and stained with Giemsa. (A) 2 hours (125X); (B) 24 hours (125X); (C) 48 hours (125X); (D) 72 hours (125X).

MGC were induced by MAb against HLA-DR antigens but not by MAb against other antigens present on monocytes (Table 4). Aggregation and fusion of monocytes to form MGC could be induced by HLA-DR specific MAb of three different isotypes. Other MAb (MHM 5) against class I MHC antigens (HLA-A,B,C) and MAb (H5A4) specific for the human monocyte-macrophage differentiation antigen HMac-1 induced cell aggregates but these did not fuse to form MGC. MAb against two other antigens expressed on monocytes, the leukocyte common antigen (H5A5) and a monocyte specific polypeptide of Mr 200,000 (H6A7), did not induce aggregation or fusion. In addition, all of the other 80+ monoclonal antibodies were negative. Control studies show that the reaction is not due to Fc receptors.

Table 4

Specificity of MAb-induced MGC

MAb	Isotype	Specificity	Aggregation	MGC Formation
MHM.33	IgG2a,k	HLA-DR	+	+
MHM.35	IgM,k	HLA-DR	+	+
MHM.36	IgG1,k	HLA-DR	+	+
MHM.5	IgG1,k	HLA-A,B,C	+	-
H5A4	IgG1,k	HMac-1	+	-
H6A7	IgM,k	p200(monocyte)	-	-
H5A5	IgG1,k	Leukocyte common	-	-

Table 4. The specificity of MAb-induced formation of MGC. Monocytes were cultured 4 days in the presence of MAb. All MAb were used in the form of immune ascites fluid at a final dilution of 1:5000. Cultures were examined for cell aggregation and fusion every 24 hours.

e. ANTIBODIES THAT INHIBIT THE MIXED LYMPHOCYTE REACTION (MLR). We recently have began to characterize two antibodies, Mon.1 and Mon.2, that act on the MLR in an apparently novel manner. These antibodies are of particular interest because they act on two antigens that are strong candidates for accessory cell molecules that stimulate T cell function. A summary of the properties of these antigens is as follows:

Mon.1 antigen of Mr 50,000: The Mon.1 monoclonal antibody (IgM,k) precipitates an antigen of Mr 50,000 that is expressed on

monocytes, macrophages, and epithelial cells. Fluorescence activated cell sorting indicated that the population of peripheral blood lymphocytes that carry the antigen is different from the T3 positive population, thus the antigen is not a T cell component.

This antibody markedly inhibited the mixed lymphocyte reaction. Surprisingly, it also enhanced the proliferative response of T cells to phytohemagglutinin (PHA). There was no cell proliferation in the absence of PHA, showing that the Mon.1 antibody is not itself mitogenic. Moreover the antibody did not affect CTL or NK activity. These results suggest that the Mon.1 antigen is involved in the induction of T cell responses, not T cell effector function. The antigen is different from all of the major proteins known to act on T cell function; i.e., T cell antigens (T3, T4, T8, T-antigen receptor, or Tac) Ia (HLA-DR), HLFA, IL-1, IL-2. Thus, The Mon.1 antigen thus appears to be a novel accessory cell protein involved in the induction of mixed lymphocyte reactions, the in vitro correlate of graft-versus-host reactions. A recent and very exciting observation is that there is markedly reduced binding of the Mon.1 antibody in the presence of anti-HLA-DR, suggesting an interaction of the Mon.1 and Class 2 MHC antigens. Further studies on the effect of the Mon.1 antibody on T cell responses in vitro should provide valuable insights into the mechanisms of lymphocyte activation.

Mon.2 antigen of Mr 80,000: The Mon.2 antibody recognizes a polypeptide expressed on the surface of all leukocytes except granulocytes. This antibody also inhibited the mixed lymphocyte reaction and enhanced the response to PHA. This was no effect on the response to soluble antigen, CTL or NK function. Although the pattern of inhibition of the Mon.2 antibody is very similar to that of Mon.1, the antigens recognized are clearly different. Possibly, the Mon.1 and Mon.2 antigens are involved in the same activation mechanism or pathway.

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