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(71) Applicant: **GENZYME CORPORATION**
One Mountain Road
Framingham, Massachusetts 01701(US)

Applicant: **BEHRINGWERKE**
Aktiengesellschaft
Postfach 1140
D-3550 Marburg 1(DE)

(72) Inventor: **Kurrle, Roland**
Schenkendorfweg 18
D-3550 Marburg(DE)
Inventor: **Shearman, Clyde W.**
10, Pheasant Hill Road
Bellingham, Massachussetts 02019(US)
Inventor: **Moore, Gordon P.**
24, Brookside Avenue
Lexington, Massachusetts 02173(US)
Inventor: **Seiler, Fritz**
Oberer Eichweg 10
D-3550 Marburg(DE)

(74) Representative: **Sheard, Andrew Gregory et al**
Kilburn & Strode 30, John Street
London WC1N 2DD(GB)

(54) Improved monoclonal antibodies against the human alpha/beta t-cell receptor, their production and use.

(57) Monoclonal antibodies against the human alpha/beta T-cell receptor are described along with pertinent sequence information of the variable and constant regions and humanised and civilised versions of such antibodies.

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IMPROVED MONOCLONAL ANTIBODIES AGAINST THE HUMAN ALPHA/BETA T-CELL RECEPTOR, THEIR PRODUCTION AND USE

The instant application discloses useful monoclonal antibodies (mAbs) against an epitope within the constant region of the human alpha/beta T-cell receptor (TCR), their production and use for immunosuppressive therapy in organ and bone marrow transplantation, in treatment of autoimmune diseases and for therapeutic applications in immunoregulation.

For decades Anti-leukocyte-antibodies for clinical and experimental use have been generated by immunizing, e.g. horses, rabbits, goats and rats with leukocytes, lymphocytes or subpopulations thereof or with various tumor cell lines. Specificity of such anti-leukocyte-globulin/Antithymocyte-globulin (ALG/ATG) preparations was usually obtained by careful selection of antigen sources or by absorption of undesired antibodies with different cell types such as erythrocytes, B-cells, selected cell lines, etc. This strategy, which resulted in high quality ALG/ATGs, requires a considerable expenditure of laboratory force, quality control, and the necessity to ascertain reproducible specificity from batch-to-batch. Within certain limitations, those ALG/ATGs made it possible to study leukocyte differentiation, the cellular origin of leukemia and lymphoma, to define T-cell subpopulations, and even to prepare antiidiotypic antisera.

The therapeutic efficacy of ALG/ATGs is well known, especially for immunosuppression in organ transplantation. In addition, ALG/ATGs have been used successfully to treat patients with aplastic anemia and for "purging" bone marrow cells within the context of bone marrow transplantation. Despite the success with ALG/ATG preparations it is accepted that even with a reasonable amount of laboratory effort, these polyclonal anti-leukocyte-antibodies might vary from batch-to-batch and the specificity of these antisera is limited.

Once the technique to produce mAbs was developed by Kohler and Milstein (Nature 225(1974)), it was possible to generate antibodies with much higher specificity as compared to ALG/ATG preparations. Since mAbs recognize not only distinct antigens, e.g. on cell surfaces, but also particular epitopes within such an antigen, they can be used with high efficacy to discriminate even between very similar cell populations, and to characterize the biochemical and functional aspects of the recognized antigen.

mAbs have been most frequently and successfully used for immunosuppressive therapy in clinical organ transplantation. However, most mAbs have a broad immunosuppressive capacity, thus undesirably influencing functions of a wide spectrum of immune cells presumably not all involved in graft rejection.

Among others, the monoclonal antibody OKT3, directed against mature human T cells, has been extensively used for the treatment of patients undergoing acute allograft rejection after kidney transplantation (Russell P.S., Colvin R.B., Cosimi, A.B.: Annu. Rev. Med. 35:63, (1984) and Cosimi A.B., Burton R.C., Colvin, R.B. et al.: Transplantation 32:535, (1981)). Moreover, OKT3 and rabbit complement were used for purging mature T-cells from donor marrow to prevent acute graf t-v-host disease (GVHD) in allogeneic bone marrow transplantation (Prentice, H.G., Blacklock, H.A., Janossy, G. et al.: Lancet 1:700, (1982) and Blacklock, H.A., Prentice, H.G., Gilmore, M.J. et al.:Exp. Hematol. 11:37, (1983)). Whereas OKT3 treatment seems to be effective in the prevention of GVHD in allogeneic bone marrow transplantation for acute leukemia, a combined in vitro/in vivo treatment with OKT3 failed to prevent GVHD during therapy for severe combined immunodeficiency (Hayward, A.R. et al.: Clin. Lab. Observ. 100:665, (1982)).

Treatment of T-cells with OKT3 elicits several responses inconsistent with immune suppression including T-cell activation, production of immune mediators and T3-modulation. The T3-antigen complex recognized by CD3-mAbs (e.g. OKT3) is postulated to play a crucial role during T-cell activation. Functional studies indicate that the T3-antigen complex is involved in specific immune functions as molecules functionally and physically associated with the respective T-cell receptor. Under physiological conditions the mere binding of OKT3 to T-cells results in T-cell activation. When T-cells are activated in the presence of accessory cells, OKT3 is highly mitogenic for T-cells from all donors, whereas for anti-T3 mAbs of the IgG1 isotype, nonresponsiveness caused by polymorphism in the accessory cell function has been described. Additionally, it has been reported that stimulation of human peripheral blood lymphocytes with OKT3 induces the production of immune mediators such as interferon (alpha-IFN) and interleukin-2 (IL-2) (Pang, R.H., Yip, Y.K., Vilcek, J.: Cell Immunol. 64:304, (1981) and Welte, K., Platzer E. Wang, C.Y., et al.: J. Immunol. 131:2356, (1983)). One of the earliest events after the binding of OKT3 to the T-cell membrane is the modulation of the T3 complex. T3 modulation occurs under appropriate conditions in vitro as well as in vivo and this mechanism, among others, seems to be responsible for the "escape" of T-cells during in vivo therapy with OKT3. Also antigenic modulation seems to play a critical role in T-cell activation.

In light of undesired effects of OKT3 described above, there was a need to create new mAbs having a specificity against mature lymphocytes and suitable for clinical application. Still another aspect and goal

involves the coupling of such mAbs to cytotoxic agents (radioisotopes, toxins, enzymes, etc.) in order to improve their effectiveness in mediating cytolysis.

Another goal involved the modification, via genetic manipulation, of the mAb in order to produce chimeric antibodies having mixed murine and human characteristics in order to improve their effectiveness and/or lower their immunogenicity in patients.

Chimeric antibodies offer an additional advantage over murine mAb with regards to immunogenicity in patients. A chimeric antibody would retain the affinity and specificity of the parental murine mAb and eliminate the patient immune response to the murine constant regions. A further refinement involves humanization of the variable regions. Only the complementarity determing regions and selected framework amino acids necessary for antigen binding are maintained murine. The remaining framework regions are converted to human sequences. The resulting mAb of the present invention is thus essentially a human antibody with a much lower immunogenicity in patients.

In accordance with the various goals, aspects and principles of the present invention, we have made a useful mAb secreted by the hybridoma cell line designated as BMA 031 by immunizing mice with human peripheral blood T-lymphocytes separated by the E-rosette-technique (so called E^+-cells). The mAb secreted by BMA 031 (in the following likewise designated as BMA 031) is a murine monoclonal antibody of the IgG2b isotype with a specificity for the alpha- and beta-chain of the TCR/CD3 receptor complex. As compared to OKT3 or BMA 030 (both clustered as CD3-antibodies), BMA 031 only very weakly induces mediator release after binding to T-cells. It is highly effective in clinical application, e.g. kidney transplant for patients with increased immunological risk when given post or at the time of the transplant-operation. Since no major side effects were observed with BMA 031 even at doses of up to 50 mg/dose, it may be advantageously given at time of surgery (preferably via a first injection of 50 mg intravenously) followed by a second injection 48 hours after transplantation. Graft function was perfect in almost all cases.

BMA 031 defines, therefore, a valuable epitope on the alpha-beta TCR distinct from the epitope on CD3 recognized by OKT3 or by other mAbs against mature T-lymphocytes. In order to completely characterize this mAb and also to permit, by recombinant DNA techniques, exchange of the variable region frameworks outside of the hypervariable regions and exchange of the human constant region in place of the endogenous murine constant region, the DNA coding for the heavy and light chains of BMA 031 was cloned and sequenced.

Principal aspects of the invention therefore involve the recognition and definition of the epitope on alpha/beta TCR and the monoclonal antibodies directed against this epitope.

Preferred embodiments of the antibodies of the present invention are BMA 031 and those mAbs containing the same amino acid sequence at the hypervariable regions, or functional equivalents thereof. These equivalents include, for example, chimeric variants in which the mouse constant regions are replaced with human C-regions.

A further aspect of the invention concerns the use of these antibodies in clinical application before, during or after transplant surgery, in bone marrow transplantation, in treatment of cancer (direct treatment of leukemic cells and indirect treatment of all types of cancer by activation of T-cell populations) and for therapeutic applications in immunoregulation.

The antibodies of the present invention are also useful in the detection of immunocompetent T-cells.

Additional aspects will become apparant upon study of the detailed description of preferred embodiments set forth below.

The description of the preferred embodiments refers by way of example to the accompanying drawings, wherein:

Figure 1 graphically depicts the construction scheme of the BMA 031 genomic library;

Figure 2 describes the probes used to screen the library;

Figure 3 graphically sets forth, in linear fashion, the human constant region expression vectors;

Figures 4A, 4B and 4C show results of competitive immunofluorescence assays with BMA 031 Chimeric Antibodies.

Figures 5A and 5B show results of T-cell proliferation assays with BMA 031 Chimeric Antibodies.

Figures 6A, 6B and 6C show results of ADCC assays with the BMA 031 Chimeric Antibodies.

Figure 7 shows the results of competitive immunofluoresence assays with BMA-EUC1V3 antibody.

As used herein, the term "epitope" refers to the structure recognized by the monoclonal antibody BMA 031, and is generally thought to be independent of the remaining portion of the antigen on which the epitope is located. It is presently unknown exactly how the epitope is formed structurally but it is anticipated that it may be formed by either (i) a part of the amino acid sequence of the antigen molecule; (ii) the three-dimensional structure formed by non-contiguous amino acids within the same molecule; (iii) the three-dimensional structure formed by various molecules within an antigen complex; or (iv) some combination

thereof. As used herein the term "monoclonal antibody (mAb)" means an antibody composition having a substantially homogeneous antibody population. It is not intended to be limited as regards to the source of the antibody or the manner in which it is made and in the most preferred embodiments is intended to include recombinant methods of manufacture.

As used herein with respect to the exemplified BMA 031 antibody, the term "functional equivalent" means a monoclonal antibody that: (a) blocks the binding of BMA 031 and whose binding is blocked by bound BMA 031; (b) binds selectively to human T-cells having expressed the alpha/beta TCR but not having expressed TCR-gamma and/or delta-chains; (c) has one of the known isotypes; (d) binds to the same antigen as BMA 031 as determined by immunoprecipitation, western blotting or other biochemical analyses; (e) binds to the same antigen as determined by cells transfected by gene(s) for the alpha/beta TCR or segments thereof.

Example 1. Immunization and cell fusion

Three Balb/c mice (female; age: 6-8 weeks) were twice immunized intraperitonally with 1.5 x 10^6 E^+-cells each. Peripheral blood human T-lymphocytes were separated by the E-rosette-technique (rosette-formation with sheep red blood cells - E^+-cells) and were derived from the peripheral blood of a healthy donor whose donated blood routinely tested HIV-negative for more than one year after having given the blood for immunization purposes.

Three days after the last immunization, the spleens of all three mice were removed, a single cell suspension was prepared and the lymphocytes were fused with the murine myeloma cell P3X63-Ag8.653 (ATCC # CRL 1580) according to a standard fusion protocol. The myeloma cell P3X63-Ag8.653 is described as an immunoglobulin non-producer mutant derived from the original myeloma cell P3-X63-Ag8 (ATCC # TIB 9).

After fusion, cells (1 x 10^5 cells/well) were cultured in the presence of HAT-medium (Dulbecco's modified Eagle's medium + 10% FCS + 0.1mM hypoxanthine, 0.4 µM aminopterin, 16 iµM thymidine) to select for hybridoma cells.

Example 2. Isolation and characterization of the hybridoma clone BMA 031

Supernatants of growing hybridoma cells (fusion number BW 242) were harvested routinely and tested for the presence of murine immunoglobulins in an ELISA test-system designed to measure murine IgG quantitatively. At the same time, the supernatants were tested for antibodies with specificity for human lymphocyte cell surface antigens.

Within this selection process, single cells were picked from the original well (BW 242/1177) and were cultured separately. In subsequent steps these cells were repeatedly cloned by micromanipulation under microscopic control (BW 242/412). During three cloning cycles 100% of growing clones produced monoclonal antibodies with identical binding specificity and identical behavior with respect to biochemical criteria. One clone was selected and designated as BMA 031.

Extensive analyses were carried out to define the specificity and the functional properties of the hybridoma clone, BMA 031.

A master seed bank and a working cell bank were established starting with the hybridoma clone BMA 031. The two cell banks underwent extensive examinations to assure absence of contamination with pathogens (mycoplasmatic, bacterial and viral infections). In addition, starting from the master seed bank, experiments were carried out which showed that even after the 50th culture passage detectable variations in antibody specificity were neither measurable nor could non-producer mutants be detected when analysed by single cell cloning and by calculating antibody production rates in bulk culture.

Example 3. Specificity of BMA 031

Monoclonal antibodies directed to lymphocyte cell surface antigens are usually characterized by a binding assay. To analyse the specificity of BMA 031, cytofluorometric assay systems were used predominantly. In particular, binding assays were carried out as described below.

3.1 Analyses of the binding of BMA 031 with peripheral blood leukocytes

Comparison of mAb reactivity with reference mAbs

Defined subpopulations of PBLs were labelled either with a reference mAb and/or with BMA 031. If both mAbs have identical specificity they will stain identical populations of cells. In these studies, the reference mAbs used were those which had been previously characterized in the Workshops for Human Leukocyte Differentiation Antigens I., II. and III. (Paris, 1982; Boston, 1984; Oxford, 1986; Bernard, A.E.: Leukocyte Typing. Springer-Verlag (1984); Reinherz, E.L.E.: Leukocyte Typing II. Springer-Verlag (1986); McMichael, A.J.E.: Leukocyte Typing III. Oxford University Press (1987).

Expression of the antigen on different leukocyte subpopulations

Peripheral blood leukocytes were analyzed on an Ortho Cytofluorograph 50H/2150 Computer-system modified for single-step analyses of whole blood. Cells were either directly labelled with BMA 031-FITC and/or with reference mAbs or alternatively stained in a second step with isothiocyanate-fluoresceinated rabbit anti-mouse IgG (Ig-F(ab')2-FITC). By using cytofluorometric assays, it was discovered that binding of BMA 031 was only detected on cells of the T-cell lineage which express the alpha/beta T-cell receptor. BMA 031 does not react with cells which express the gamma/delta T-cell receptor. BMA 031 can therefore effectively be used to discriminate between alpha/beta-TCR^+ and gamma/delta-TCR^+ cells. Presence or absence of distinct TCR chains reflect the status of T-cell differentiation during T-cell ontogeny. In peripheral blood, molecules of the CD3-complex are predominantly expressed in association with the human alpha/beta-TCR (7). In healthy blood donors, the frequency of T-lymphocytes stained by BMA 031 is usually only 1-5% lower than that measured with CD3 mAbs (for normal frequencies of $CD3^+$ cells see 1-3). As shown with cloned T-cells, this population of $CD3^+$ BMA 031^- cells express the gamma/delta TCR instead of the alpha/beta TCR. In pathological situations, however, the frequency of $CD3^+$ BMA 031^- T-cells can increase to 20% of the $CD3^+$ cells in individual patients. On $CD3^+$ BMA 031^+ cells, binding of BMA 031-FITC is blocked by OKT3 and vice versa in competitive immunofluorescence assays. Nevertheless, by analyzing such data in more detail (comparison of fluorescence-histograms) and by blocking studies with anti-idiotypic antisera it can be clearly shown that BMA 031 recognizes a different epitope than all known CD3-mAbs.

Example 4. Characterization of the functional properties of BMA 031

BMA 031 is a murine monoclonal antibody of the IgG2b isotype. Due to the unique specificity and the isotype of BMA 031, this antibody triggers a specific pattern of biological functions. After binding to T-cells, BMA 031 does not induce T-cell proliferation comparable to CD3-mAbs of the IgG2a isotype (e.g. BMA 030, OKT3) in a three day thymidine incorporation assay nor does it induce Ca^{2+} influx in resting T-lymphocytes . In contrast to CD3-mAbs such as BMA 030 or OKT3, binding of BMA 031 to T-cells induces antigen modulation only weakly and triggers the release/production of cytokines to a very low extent.

Example 5. Preparation of DNA and RNA from BMA 031

For preparation of genomic DNA, approximately 1 x 10^9 cells were grown in T-flasks. DNA was prepared by lysis in SDS, digestion with proteinase K and RNAse A and sequential, gentle, phenol/chloroform extractions in high salt. Low density agarose gels indicated that the average length of the genomic DNA was greater than 50 kilobases (Kb); long length is important in creation of complete genomic clone libraries in lambda phage vectors. The yield was about 10 mg of DNA.

Approximately 10^9 cells were also grown for RNA isolation. Cells were lysed and RNA extracted using guanidinium thiocyanate. RNA yield was about 10 mg and it appeared clean and undegraded on agarose gels. Poly A^+ RNA was prepared by binding total RNA to oligo dT cellulose. Yield was about 5%, i.e. about 500 µg of poly A^+ mRNA.

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