*Biotecnología Aplicada, Vol. 7, No. 3, 282-289 (1990)*

# Cloning of the Light and Heavy Chain Variable Regions of the Anti-CD6 IOR-T1 Mouse Monoclonal Antibody Using the Polymerase Chain Reaction

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*Recibido en febrero de 1990*
*Aprobado en abril de 1990*

## SUMMARY

IOR-T1 is a mouse anti-CD6 monoclonal antibody (MAb) with antitumoral effect *in vivo* against cutaneous T-cell lymphomas. With the ultimate goal of producing genetically engineered chimeric antibodies with increased effectiveness in human treatment, we report the cloning of the IOR-T1 variable regions using of the polymerase chain reaction (PCR) and primers designed for conserved sequences of framework one and constant regions of mouse immunoglobulin light and heavy chain genes. RNA was extracted from the mouse hybridoma cells secreting MAb IOR-T1, and first strand cDNA was synthesized. The light and heavy chain variable regions were amplified using PCR and synthetic oligonucleotides. Isolated PCR light and heavy chain DNA fragments were sequenced after M13 cloning. The IOR-T1 variable regions can be ascribed to the subgroups V (light chain), and "miscellaneous" (heavy chain), defined by Kabat *et al.*, (1987). These methods greatly facilitate structural and functional studies of antibodies by reducing the efforts to clone and sequence the members of the immunoglobulin multigene family.

## RESUMEN

El IOR-T1 es un anticuerpo monoclonal (AcM) de ratón contra la molécula CD6, cuyo efecto antitumoral en los linfomas cutáneos de tipo T ha sido demostrado recientemente en ensayos clínicos. Con el objetivo final de producir por ingeniería genética anticuerpos quiméricos que posean una mayor efectividad en el tratamiento del humano, nosotros hemos clonado las regiones variables del AcM IOR-T1 empleando la reacción en cadena de la polimerasa (PCR) y cebadores diseñados para su hibridación con secuencias conservadas de las regiones "marco uno" y constante de los genes que codifican para las cadenas ligera y pesada. Para ello, se extrajo ARN del hibridoma secretor de IOR-T1 y se sintetizó cADN de simple cadena. Las regiones variables de las cadenas ligera y pesada se amplificaron usando PCR y oligonucleótidos sintéticos. El ADN de cadenas ligera y pesada se clonó en M13 y secuenció subsecuentemente. Las regiones variables del AcM IOR-T1 pueden ser acotadas en los subgrupos génicos V (cadena ligera) y "miscelánea" (cadena pesada), según la clasificación de Kabat *et al.* (1987). Estos métodos facilitan grandemente los estudios estructurales y funcionales de los anticuerpos, reduciendo los esfuerzos para clonar y secuenciar miembros de la familia multigene de las inmunoglobulinas.

## INTRODUCTION

The effectiveness and safety of mouse monoclonal antibodies (MAbs) in human therapeutics (Larrick and Bourla, 1986) can be affected by the fact that murine MAbs induce a human immune response (HAMA) in many patients (Shawler *et al.*, 1985; Schroff *et al.*, 1985). Using recombinant DNA technology it is now possible to engineer antibodies with a number of defined characteristics such as isotype, size,

domain structure, carbohydrate addition sites, etc.(see general reviews in Williams 1988, and Morrison and Oi, 1989). Hence efforts have been directed to the construction of chimeric mouse/human MAbs in the hope that replacement of all but the murine variable regions or complementarity determining regions (CDRs) with human sequences will reduce the HAMA response (Liu *et al.*, 1987; Steplewski *et al.*, 1988; Sun *et al.*, 1987; Beidler *et al.*, 1988; Riechmann *et al.*, 1988; LoBuglio *et al.*, 1989; Hale *et al.*, 1988).

Until recently, obtaining the variable region sequence had been the limiting step in rapidly constructing recombinant antibody molecules, but the introduction of the polymerase chain reaction (PCR; see review in Oste, 1988) for the rapid direct cloning and sequencing of human and mouse immunoglobulin variable region genes (Larrick et al 1989; Orlandi *et al.*, 1989; Gavilondo *et al.*, 1990) has greatly simplified the technique.

IOR-T1 is a mouse anti-CD6 monoclonal antibody (MAb) shown to be effective *in vivo* against cutaneous T cell lymphomas (García *et al.*, 1990). With the ultimate goal of producing genetically engineered chimeric antibodies for human treatment, we have cloned the light and heavy chain variable region genes of IOR-T1, using PCR and primers designed for conserved sequences of framework one (FR1) and constant (CONST) regions. The base sequences of the relevant light and heavy chain variable regions are presented.

## MATERIALS AND METHODS

### Cell line

The mouse hybridoma cell line 43/27/F6 secretes the IgG2a(k) MAb denominated IOR-T1 that recognizes the CD6 (120 kd) molecule present in human T lymphocytes (García *et al.*, 1984; García *et al.*, 1990). Cells were grown in RPMI 1640 culture medium (GIBCO, Grand Island, NY), with 10% newborn calf serum (Cubavet, La Habana), supplemented with 2 mM glutamine, 50 uM 2-mercaptoethanol, 0.48 mM sodium pyruvate, 0.17 uM bovine insulin and 1.3 mM cis-oxaloacetic acid (GIBCO, Grand Island, NY).

### Oligonucleotide primer design

Oligonucleotide PCR primers were designed using the database of Kabat et al (1987), and Genbank. The 5' primers were constructed from information available on conserved sequences of the first framework (FR1) regions of light and heavy chains (see denomination "FR1..." in Table I). The 3' primers were designed for annealing within the constant regions of the light kappa (Ck) and heavy (CH1: first gamma domain) mouse chains (see denomination "CONST..." in Table 1). The primers had EcoRI or Hind III restriction sites on the 5' and 3' ends, respectively. Two extra bases (Gs) were added outside the restriction site to improve enzyme digestion. Oligonucleotides were made on a Gene Assembler Plus DNA synthesizer (Pharmacia-LKB, Uppsala). See Figure 1 for a graphic representation of priming sites.

### Preparation of RNA

RNA was extracted with the technique suggested by Gough (1988); $10^5$ hybridoma cells were used as starting material.

### First strand synthesis

First strand DNA synthesis was performed using the Boehringer-Mannheim (Chicago, IL) cDNA kit; briefly, total RNA samples (approximately 0.5 $\mu$g; derived from $10^5$ hybridoma cells) were heated at 65°C for one hour, and incubated with a mixture of RNase inhibitor, deoxynucleotides, oligo $(dT)_{15}$ as primer, and AMV reverse transcriptase, for 60 minutes at 42°C.

### Polymerase chain reaction

Eighty ul of PCR mix were added to the 10 ul of first strand cDNA. The PCR mix was made following the instructions of the Perkin-Elmer Cetus (Norwalk, CT) PCR kit. Five ul of each primer were added to give a final primer concentration of 1 uM and the mixture was subjected to PCR amplification using the Hybaid (UK) thermal cycler set, for 30 cycles. The temperatures and times used for PCR were: melting at 94°C, 1 minute; primer annealing at 55°C, 1 minute; primer extension at 72°C, 1 minute.

**Table 1**
**DESIGN OF THE 5' AND 3' END SYNTHETIC PRIMERS FOR PCR**

MOUSE LIGHT KAPPA CHAINS:

**5': EcoRI/FR1-ML(kappa) (aminoacids 1-8):**
5'---GGGAATTCGA(CT)ATTGTG(AC)T(AG)AC(AC)CA(AG)(GT)(AC)TCAA---3'

**3': HindIII/CONST-ML(kappa) (aminoacids 116-122)::**
5'---GGAAGCTTACTGGATGGTGGGAAGATGGA----3'

MOUSE HEAVY GAMMA CHAINS:

**5': EcoRI/FR1-MH (aminoacids 1-8):**
5'---GGGAATTC(GC)AGGTG(AC)AGCTC(GC)(AT)(AG)(CG)A(AG)(TC)C(CG)GGG---3'

**3': HindIII/CONST-MH(gamma) (aminoacids 121-130):**
5'---GGAAGCTTA(TC)CTCCACACACAGG(AG)(AG)CCAGTGGATAGAC---3'

Notes: Bases in parentheses represent substitutions at a given position i.e. (AT) means both A and T were present in equimolar amounts during the synthesis of a particular position. EcoRI (5' end) and HindIII (3' end) sites are underlined.

Normally one minute ramp times were used between these temperatures. Ethidium bromide stained 2% agarose (NuSieve) gels were used to visualize PCR fragments.

### M13 cloning

Gel purified heavy chain DNA PCR products (NA45 paper, Schleicher & Schuell, Keene, NH) were digested with EcoRI and HindIII restriction enzymes and ligated into M13mp18/19 sequencing vectors (Messing *et al.*, 1977; Vieira and Messing, 1982).

### DNA sequencing

Dideoxynucleotide chain termination sequencing was carried out using the Sequenase 2.0 kit from United States Biochemical Corp.(Cleveland, OH) according to the manufacturer's protocol, with $^{35}$S-$\alpha$ATP (Amersham). Two independent PCR samples and/or at least two independent M13 clones were used to validate the sequence.

## RESULTS AND DISCUSSION

Cutaneous T-cell lymphomas (CTCL) are neoplastic diseases characterized by malignant T-lymphocyte skin infiltrates, with a poor response to conventional therapy. The IOR-T1 MAb identifies a majority of CTCL malignant T-lymphocytes, and does not induce the modulation of the antigen. In recent controlled pilot studies and clinical trials it was shown that the topical or intravenous (i.v.) application of IOR-T1 leads to dramatic regressions of skin plaques and lesions in CTCL patients (García *et al.*, 1990; Gavilondo, 1990). As HAMA has been also documented in some of the i.v.-treated patients, it is our present interest the production of genetically

engineered chimeric IOR-T1 antibodies for *in vivo* use, and in this article we report our work in the first step for such task, e.g. the cloning of the IOR-T1 variable regions.

As the cloning of variable regions of rearranged immunoglobulin genes by conventional techniques is often time- and effort-consuming, we have employed PCR and specially designed primers (see Figure 1). The 5' end primers for FR1, and 3' end primers for Ck and CH1, were designed taking as basis conserved sequences available in databases. Our 5' end FR1 primers hybridize with the first 8 aminoacids of the mouse light or heavy chain variable regions. Both FR1 oligonucleotides have important differences in base composition, and degeneracy, with respect to those reported by Orlandi *et al.* (1989) for similar purposes. On the other hand, the 3' end primers hybridize in constant domains, instead of using the FR4 sites employed by Orlandi *et al.* (1989); we consider this latter approach as possibly advantageous both because of the high degree of conservation found in these regions, as well as due to the fact that the oligonucleotide structure will not interfere with the actual sequence of the variable region, thus avoiding any potential change in the spatial projection of the relevant complementarity determining regions three (CDR3s).

As sequence conservation for one same region is relative among different antibodies, it was necessary to include degenerate base positions in three of our primers. Figure 2 shows the PCR amplification products for the IOR-T1 light and heavy chain variable regions, using the FR1/CONST primer combinations. The major amplified cDNA fragments for light and heavy chains were ≈390 and ≈420 bp in size, respectively, explained by the 3' priming in the constant regions, and the presence of attached restriction sites in our oligonucleotides. These results indicate that for mouse cDNA, degenerate oligonucleotide primers will nevertheless prime the PCR reaction; this

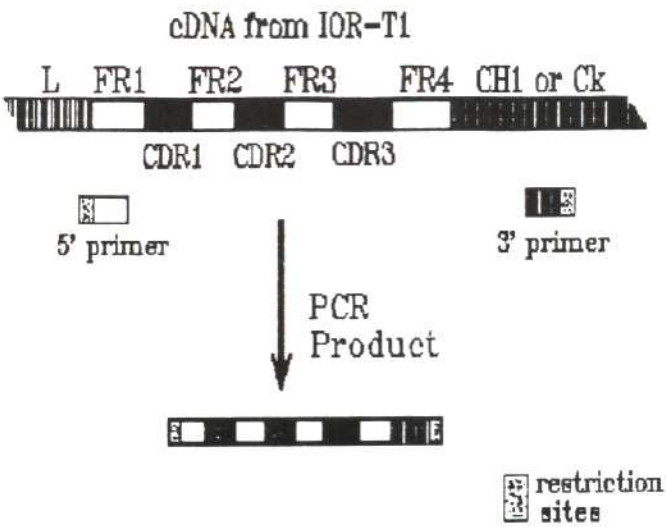


FIG. 1. Strategy for the PCR cloning of mouse rearranged immunoglobulin variable light and heavy chain regions. Priming sites for the EcoRI/FR1 and HindIII/CONST synthetic oligonucleotides are shown. L: leader sequence.

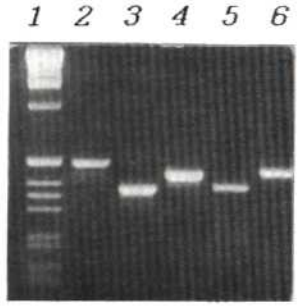


FIG. 2. Immunoglobulin light and heavy chain PCR products derived from first-strand cDNA. Ten microliters from a 100 microliter PCR reaction were applied to a 2% agarose gel for electrophoresis in TBE buffer. The major PCR products obtained after ethidium bromide staining are shown. LANE 1:1 kb DNA marker ladder; LANE 2: Perkin Elmer Cetus kit control (500 bp); LANES 3 and 5: cDNA from IOR-T1 amplified with the light chain EcoRI/FR1-ML(kappa) and HindIII/ML(kappa)-CONST primers; LANES 4 and 6: cDNA from IOR-T1 amplified with the heavy chain EcoRI/FR1-MH and HindIII/CONST-MH(gamma) primers.

fact had already been pointed out before by Larrick *et al.* (1989) in the case of human immunoglobulin region amplification, and suggests strongly that PCR can tolerate a number of mismatches and still obtain consistent priming of the polymerase extension reaction.

The sequences of the IOR-T1 light and heavy chain variable regions were obtained by M13 cloning of major PCR bands originated with our primer design, and are shown in Figures 3 and 4. The IOR-T1 variable regions belong to the immunoglobulin mouse gene subgroups V (light chain) and "miscellaneous" (heavy chain) described by Kabat *et al.* (1987).

The problems associated with the design of "universal" primers for PCR cloning of immunoglobulin variable region genes have been discussed in some recent publications (Larrick *et al.*, 1989; Orlandi *et al.*, 1989; Sastry *et al.*, 1989; Ward *et al.*, 1989). In principle, one must consider that variability in sequence exists among antibodies, even for the most conserved regions; also, differences in functioning of the primers could depend on the source of DNA or cDNA (i.e. from hybridoma cells or stimulated lymphocytes). Finally, unexpected homologies between different framework regions of one same chain could be present (in fact, we have recently shown that the particular design of the 5' light chain primer of Orlandi *et al.* (1989) can produce undesirable annealing possibilities with the framework two (FR2) region of some mouse kappa II light chain genes (Gavilondo *et al.*, 1990).

Our FR1/CONST primer design has led also to specific amplifications of mouse light chains of the kappa types II, V and VI, and heavy chain gamma gene families IIc and IIb (Larrick *et al.*, 1990; Gavilondo *et al.*, 1990). Light and heavy chains (still unclassified) from two other hybridomas and one myeloma have also been amplified to adequate size fragments using these primers. On such basis, our oligonucleotide design can hopefully be used for the cloning and subsequent sequencing of many mouse hybridomas.

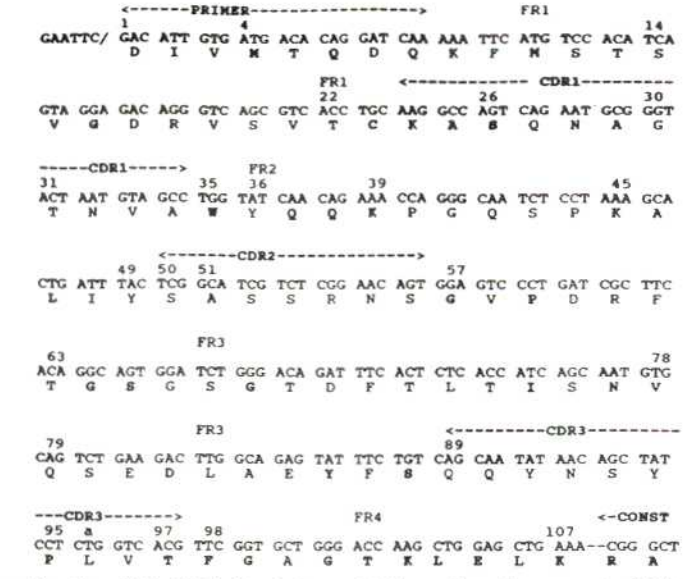


FIG. 3. Sequence for the IOR-T1 light chain variable region. Framework (FR), and complementarity determining regions (CDRs) are indicated. Amino acids reported as "invariant" in the database of Kabat *et al.* (1987) for mouse light chains V subgroup are in bold. 5' primer site, and first aminoacids of the kappa constant (CONST) domain are indicated.

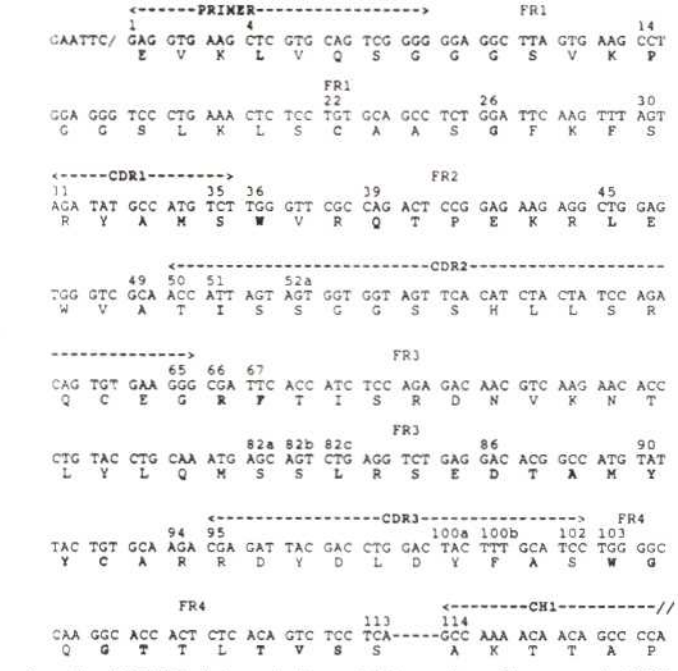


FIG. 4. Sequence for the IOR-T1 heavy chain variable region. Framework (FR), and complementarity determining regions (CDRs) are indicated. Amino acids reported as "invariant" in the database of Kabat *et al.* (1987) for mouse heavy chains "miscellaneous" subgroup are in bold. 5' primer site, and first aminoacids of the gamma CH1 domain are indicated.

## ACKNOWLEDGEMENTS

We thank Victor Jiménez for his help with oligonucleotide synthesis. This work was partially supported by a grant from the National Swedish Agency for Research Cooperation (SAREC).

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