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Molecular Mechanisms for B Lymphocyte Selection: Induction and Regulation of Antigen-Receptor-Mediated Apoptosis of Mature B Cells in Normal Mice and their Defect in Autoimmunity-Prone Mice

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Molecular mechanisms for B lymphocyte selection: induction and regulation of antigen-receptor-mediated apoptosis of mature B cells in normal mice and their defect in autoimmunity-prone mice

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SUMMARY

Apoptosis (programmed cell death) has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. By crosslinking the antigen receptor (surface immunoglobulin; sIg) on the peritoneal B cells of normal mice, we found that strong crosslinking of sIg induces apoptosis of mature B cells, suggesting that interaction with membrane-bound self-antigens may eliminate self-reactive mature B cells by apoptosis. Antigen-receptor-mediated B cell apoptosis is blocked when a signal is transduced via the CD40 molecule on the B cell surface. Because the ligand of CD40 (CD40L) is expressed on activated T helper cells, B cells may escape from apoptosis and are activated when the immune system interacts with foreign antigens, which are normally able to activate T helper cells. Moreover, sIg crosslinking fails to induce apoptosis of both bcl-2-transgenic mice and autoimmune-disease-prone New Zealand mice. In these mice, the defect in sIg-mediated apoptosis of mature B cells may allow generation of self-reactive B cells, resulting in pathogenic consequences.

1. INTRODUCTION

Since Chiller *et al.* (1970) first demonstrated that tolerance can be induced within the B cell compartment, tolerance of self-reactive B cells has been suggested to be involved in the prevention of autoimmunity in the immune system. Self-tolerance of B cells may be maintained by elimination (clonal deletion) or functional inactivation of self-reactive B cells (clonal anergy) (Nossal 1983). Both of these mechanisms have been clearly demonstrated in autoantibody-transgenic mice, in which almost all the B cells express exogenous autoantibody genes and thus react to given self-antigens (Goodnow 1992). In autoantibody transgenic mice, self-reactive B cells are tolerated at the immature stage in the bone marrow. Immature B cells are thus likely to be the targets of the self-tolerance mechanisms: when self-reactive B cells in the bone marrow interact with the self-antigens, a signal via the antigen receptor (surface immunoglobulin; sIg) may either inactivate or eliminate self-reactive immature B cells. This assumption agrees with previous findings that immature B cells in the foetus, neonates or adult bone marrow are tolerated more easily than mature B cells in the peripheral lymphoid organs. Recent studies on autoantibody-transgenic mice, however, have demonstrated that self-reactive B cells in the periphery are also tolerated upon interaction with self-antigens (Goodnow *et al.* 1989; Russel *et al.* 1991). This observation suggests that sIg can transduce a tolerogenic signal in mature B cells, although signalling through sIg most likely initiates an antibody response to foreign antigens by activating mature B cells. Here we demonstrate that signalling through sIg is able to induce apoptotic cell death of mature B cells in the peritoneal cavity and discuss the mechanisms determining the fate (activation versus cell death) of mature B cells stimulated by antigens.

2. ANTIGEN-RECEPTOR-MEDIATED APOPTOSIS OF MATURE B CELLS

In mice transgenic for anti-red blood cell (RBC) autoantibody, numbers of B cells are markedly reduced in the bone marrow, spleen, lymph nodes and peripheral blood (Okamoto *et al.* 1992). This observation indicates that RBC-reactive B cells are deleted upon interaction with self-antigens (RBC). However, we found normal numbers of B cells in the peritoneal cavity of the transgenic mice. Interestingly, almost all of the peritoneal B cells in the transgenic mice are B-1 cells, which constitute a distinct B cell subset from conventional B cells, whereas the

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peritoneal cavity of normal mice contain both conventional B and B-1 cells. Several lines of evidence suggest that a small number of B-1 cells which, by a yet to be unidentified mechanism, have escaped from clonal deletion, migrate to the peritoneal cavity and extensively expand there (Murakami *et al.* 1992). Unlike conventional B cells, B-1 cells are able to expand in the peritoneal cavity because they possess self-replenishing capacity. Moreover, sequestration of peritoneal B cells from the RBC self-antigen seems to be essential for the expansion of self-reactive B-1 cells. Indeed, intraperitoneal injection of RBC results in massive apoptotic cell death of peritoneal B-1 cells in transgenic mice.

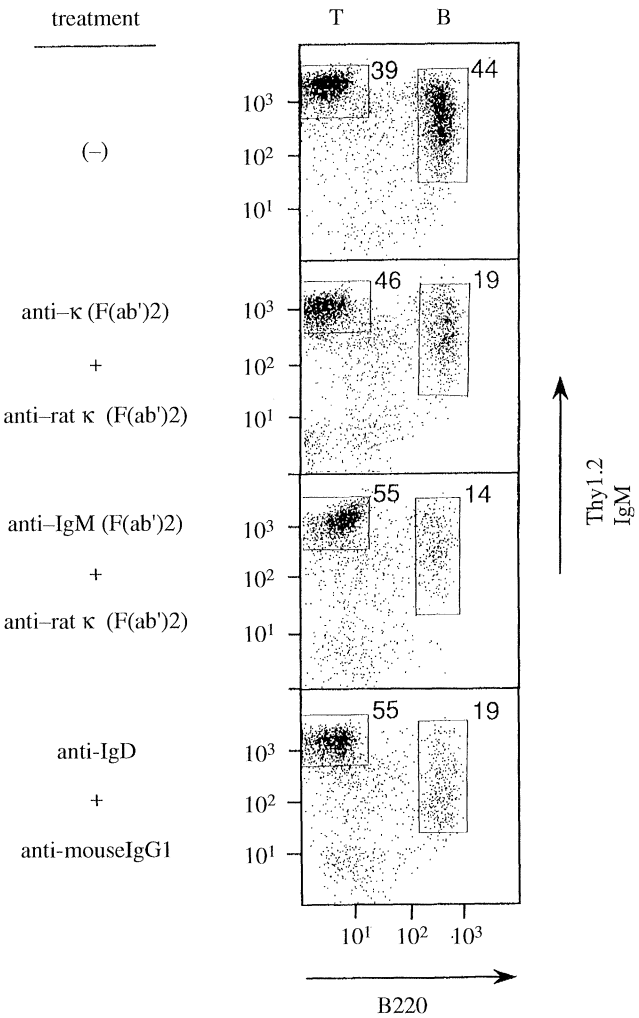

Figure 1. Signalling through sIg eliminates peritoneal B cells *in vivo*. C57BL/6 mice (6 week old) were injected intraperitoneally with 200 μg of the $F(ab')_2$ fragments of either R33-18 (rat anti-mouse κ) or Ak9 (rat anti-mouse IgM) followed by the injection of 200 μg of $F(ab')_2$ fragments of MAR18.5 (mouse anti-rat κ). Alternatively, C57BL/6 mice at the same age were injected intraperitoneally with the combination of 414/D7 (anti-mouse IgD^b, mouse IgG_1) and Ig(4a)10.9 (anti-mouse IgG_1^a). After 12 h, peritoneal cells were recovered, stained for Thy1, B220 and IgM, and analysed by two colour flow cytometry using a FACScan. Small lymphocytes were gated by forward versus side scatter. Percentages of B and T cell populations are indicated. Note that, by anti-Ig treatment, percentages of B cells are markedly reduced, whereas those of T cells are rather increased, suggesting that anti-Ig specifically eliminates B cells. Taken from Tsubata *et al.* (1994) with permission.

This finding indicates that signalling through the antigen receptor induces apoptosis of self-reactive mature B cells in these transgenic mice.

To determine whether signalling through sIg induces apoptosis of normal B cells, we injected anti-Ig antibodies into the peritoneal cavity of 6–8 week old normal C57BL/6 mice (Tsubata *et al.* 1994). We crosslinked sIg of peritoneal B cells by intraperitoneal injection of 200 μg each of anti-Ig and a second antibody which binds to the anti-Ig. Both B-1 cells and conventional B cells in the peritoneal cavity of the normal mice underwent apoptosis within 12 h (figures 1 and 2). As treatment with $F(ab')_2$ preparations of anti-Ig and the second antibody also induced apoptosis of peritoneal B cells, this reaction does not require signalling through the Fcγ receptor, which has been shown to transduce a negative signal in B cells. In contrast, injection of smaller amount of $F(ab')_2$ preparation of anti-Ig alone does not induce apoptosis but enhances class II MHC expression of peritoneal B cells, suggesting that this treatment induces signalling through sIg for B cell activation. This result agrees with previous findings that anti-Ig treatment activates mature B cells in both *in vitro* (Parker 1980; DeFranco *et al.* 1982) and *in vivo* systems (Finkelman *et al.* 1982), although proliferation and differentiation of the activated B cells require T cell-derived cytokines. Taken together, strong sIg crosslinking eliminates normal mature B cells by apoptosis, whereas weaker sIg crosslinking activates mature B cells. It is, however, not yet clear whether mature B cells in lymphoid organs other than the peritoneal cavity undergo apoptosis by sIg crosslinking. Because membrane-bound antigens crosslink sIg more strongly than soluble antigens, interaction with membrane-bound antigens may induce apoptosis of normal mature B cells as well as B cells in anti-RBC transgenic mice. Soluble antigens, in contrast, may activate responding B cells.

3. REGULATION OF ANTIGEN-RECEPTOR-MEDIATED APOPTOSIS OF B LYMPHOCYTES

The finding that strong sIg crosslinking induces apoptosis of mature B cells made us ask how the immune system responds to membrane-bound foreign antigens such as antigens on the bacterial wall. We examined the molecular mechanisms that inhibit antigen-receptor-mediated B cell apoptosis.

The bcl-2 proto-oncogene was originally identified because of its frequent translocation in follicular lymphomas (Bakhshi *et al.* 1985; Cleary & Sklar 1985; Tsujimoto *et al.* 1985). Recent studies have revealed that expression of bcl-2 rescues both hemopoietic cells and nervous cells from apopotosis in many, but not all, experimental systems (Vaux *et al.* 1988; Hockenberry *et al.* 1990; Nunez *et al.* 1990; Garcia *et al.* 1992; Allsopp *et al.* 1993). We thus injected anti-Ig and the second antibody into the peritoneal cavity of bcl-2-transgenic mice, which produce a large amount of bcl-2 in B cells. Strong sIg crosslinking does not induce apoptosis but activates peritoneal B cells of bcl-2 transgenic mice (Tsubata *et al.* 1994). This observation suggests

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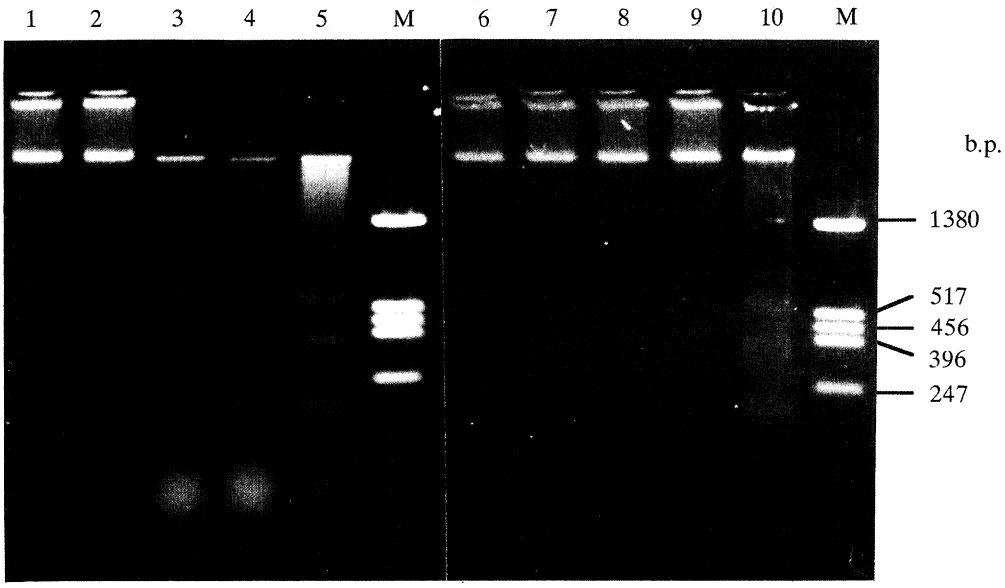

Figure 2. DNA fragmentation assay of peritoneal B cells of 6 week old C57BL/6 mice. DNA was extracted from the peritoneal cells of non-treated mice (lanes 1 and 6), mice treated with rat anti-mouse κ (187.1) (lanes 3 and 8) or with anti-rat IgG (purchased from Zymed) (lanes 4 and 9), mice intraperitoneally injected with both rat anti-mouse B220 (RA3-6B2) and anti-rat IgG (lanes 2 and 7) and mice treated with both anti-κ and anti-rat IgG (lanes 5 and 10). Treatment of mice was done as indicated in the legend to figure 1. Low molecular mass genomic DNA (lanes 1–5) and high molecular mass DNA (lane 6–10) was prepared and analysed by agarose gel electrophoresis. Molecular mass markers (M) in base pairs (b.p.) are indicated. Note that treatment with the combination of anti-κ and the second antibody (anti-rat IgG) induced DNA fragmentation, a marker for apoptosis, whereas the treatment with either anti-κ alone, the second antibody alone or the combination of the control antibody (anti-B220) and the second antibody failed. Taken from Tsubata *et al.* (1994) with permission.

that, in the presence of rescue molecules like bcl-2, B cells are able to respond to membrane-bound antigens.

As is the case for normal peritoneal B cells, WEHI-231 B lymphoma cells undergo apoptosis upon sIg crosslinking by anti-Ig (Benhamou *et al.* 1990; Hasbold & Klaus 1990). Scott *et al.* (1987) demonstrated that in the presence of T helper cells, WEHI-231 cells are resistant to anti-Ig treatment. T helper cells may express the molecule(s) which induce(s) B cells to express molecules protecting the cells from apoptosis. To determine the molecules involved in the rescue of WEHI-231 cells from apoptosis, we compared T helper lines capable of blocking apoptosis of WEHI-231 cells and those incapable (Tsubata *et al.* 1993). We found that T helper lines having the rescuing ability of WEHI-231 cells from apoptosis expressed the ligand for the B cell antigen CD40 (CD40L) (Clark & Ledbetter 1986; Stamenkovic *et al.* 1989; Armitage *et al.* 1992); those without the rescuing ability did not. We then demonstrated directly the involvement of CD40-mediated signalling in rescuing anti-Ig-treated WEHI-231 cells by treating WEHI-231 cells with the anti-CD40 antibody, soluble chimeric molecules containing the active portion of CD40L or CD40L-transfected cells. Any of these treatments protected WEHI-231 cells from antigen-receptor-mediated apoptosis. Furthermore, WEHI-231 cells upregulate class II MHC expression when treated with both anti-Ig and anti-CD40, indicating that B cells are activated in the co-existence of antigens and T-helper-cell-derived signals. Taken together, B cells may undergo apoptosis upon interaction with membrane-bound antigens alone, and require a T-helper-cell-derived rescue signal for responding to the membrane-bound antigens. This model is in agreement with the two-signal model for B cell activation originally proposed by Bretcher & Cohn (1970). As CD40L is expressed on activated T helper cells but not resting T cells (Lane *et al.* 1992; Noelle *et al.* 1992), B cells are most likely rescued when the antigens are able to stimulate T cells as well as B cells. Antigen-receptor–mediated apoptosis may thus eliminate self-reactive B cells generated within the mature B cell compartment because self-reactive T cells are eliminated in the thymus. It is of note that mature T cells do not change their antigen specificity because they do not undergo somatic mutation of the antigen receptor genes. In contrast, membrane-bound foreign antigens stimulate both T and B cells, resulting in rescuing antigen-stimulated B cells from apoptosis by signalling through CD40.

4. DEFECT IN ANTIGEN-RECEPTOR-MEDIATED APOPTOSIS IN AUTOIMMUNITY

Although self-reactive B cells may be tolerated at the immature B cell stage, self-tolerance mechanisms may also be required at the mature B cell stage. Indeed, self-reactive B cells are generated within the mature B cell compartment from B cells that have no self-reactivity by somatic mutations of Ig V genes (Diamond & Scharff 1984; Giusti *et al.* 1987). Furthermore, self-reactive B cells reacting to the self-antigens

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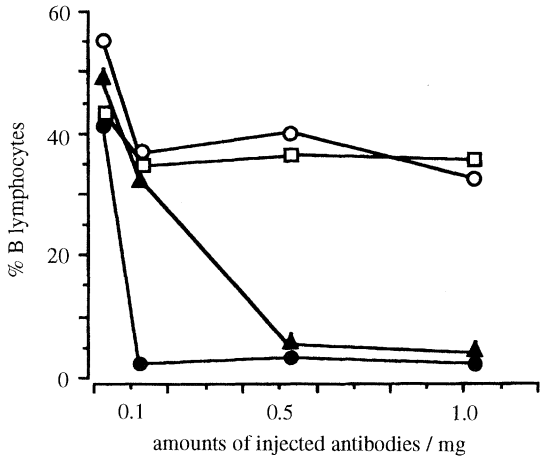

Figure 3. Failure of sIg-mediated apoptosis of peritoneal B cells in autoimmunity-prone NZB and (NZB × NZW)F1 mice. Autoimmunity-prone NZB (open circles) and (NZB × NZW)F1 (open squares) mice as well as normal C57BL/6 (filled circles) and NZW (filled triangles) mice (6 week old) were injected intraperitoneally with 100, 500 or 1000 μg of MB86 (anti-mouse IgM^b) and the same amount of the second antibody (goat anti-mouse IgG1). After 12 h, the peritoneal cells were collected and stained for B220 and IgM. Percentages of B cells ($B220^+$, IgM^+) were determined by flow cytometry as described in the legend to figure 1. Taken from Tsubata *et al.* (1994) with permission.

localized in the periphery are not eliminated at the immature stage in the bone marrow. Those self-reactive mature B cells may be eliminated upon interaction with self-antigens. Defects in this mechanism may thus allow self-reactive B cells to survive, resulting in autoantibody production.

Anti-DNA antibodies are characteristic of systemic lupus erythematosus (SLE), a prototypic autoantibody-mediated autoimmune disease. B cells reactive to DNA have been shown to be either eliminated or functionally inactivated in the anti-DNA-transgenic mice of the normal background, suggesting that anti-DNA B cells are tolerated in normal mice. In contrast, a large amount of anti-DNA antibodies is produced in bcl-2-transgenic mice, which also develop SLE-like immune complex nephritis (Strasser *et al.* 1991). This finding suggests some defect in the self-tolerance mechanisms in the bcl-2-transgenic mice. Indeed, peritoneal B cells do not undergo apoptosis upon anti-Ig injection, suggesting that bcl-2 transgenic mice fail to eliminate self-reactive mature B cells (figure 4). However, Goodnow's group (Hartley *et al.* 1993) and ours (Nisitani *et al.* 1993) have shown that bcl-2 does not block clonal deletion of self-reactive B cells at the immature stage in the bone marrow. Self-reactive B cells may thus emerge within the mature B cell compartment in the bcl-2 transgenic mice presumably due to the defect in antigen-receptor-mediated apoptosis. This finding also suggests the importance of self-tolerance mechanisms in the mature B cell compartment in protecting normal individuals from autoimmunity.

F1 hybrids of NZB and NZW (BWF1) mice produce anti-DNA antibody and develop nephritis spontaneously. NZB mice develop autoimmune hemolytic anemia by producing anti-RBC autoantibodies. To examine the B cell tolerance mechanisms of these autoimmune-disease-prone mice, we treated 6–8 week old NZB and BWF1 mice with intraperitoneal injection of anti-Ig, followed by the injection of the second antibody (Tsubata *et al.* 1994). In both strains of mice, percentages of B cells in the peritoneal cells are only slightly reduced even by the injection of 1 mg each of anti-Ig and the second antibody (figure 3). In contrast, almost all the B cells undergo apoptosis by the injection of 200 μg each of anti-Ig and the second antibody in normal C57BL/6 mice. Slight reduction in

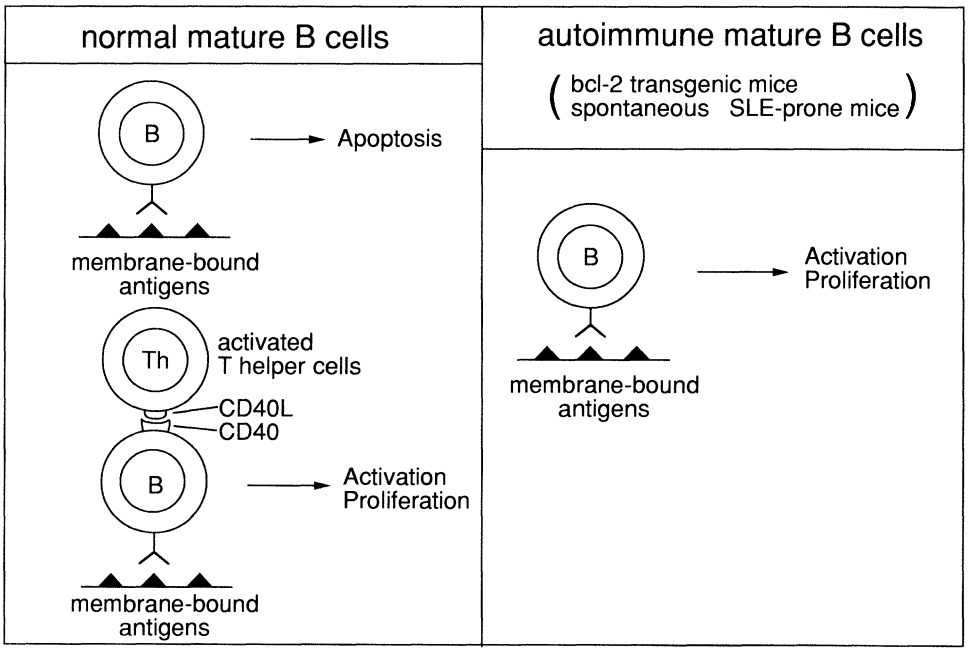

Figure 4. Model of activation and apoptosis of B cells upon interaction with membrane-bound antigens. Taken from Tsubata *et al.* (1994) with permission.

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B cell percentage in the autoimmunity-prone mice may be due to the expansion of non-lymphocytic inflammatory cells by anti-Ig injection. Mature B cells of the autoimmunity-prone mice are likely to be resistant to antigen-receptor-mediated apoptosis and to allow self-reactive B cells to survive (figure 4). It has been demonstrated that high-affinity anti-DNA antibodies, which are presumably pathogenic, are produced by extensive somatic mutations of Ig V genes (Hirose *et al.* 1993). This result suggests that those pathogenic autoantibodies are generated in the mature B cell compartment as is the case for bcl-2-transgenic mice. Although, we failed to detect enhanced expression of bcl-2 in the autoimmunity-prone mice, those mice may produce an excess amount of bcl-2-related molecules such as bcl-x (Boise *et al.* 1993) or Mcl-1 (Kozopas *et al.* 1993). Molecular mechanisms responsible for the resistance of NZB and BWF1 B cells to apoptosis are now under investigation.

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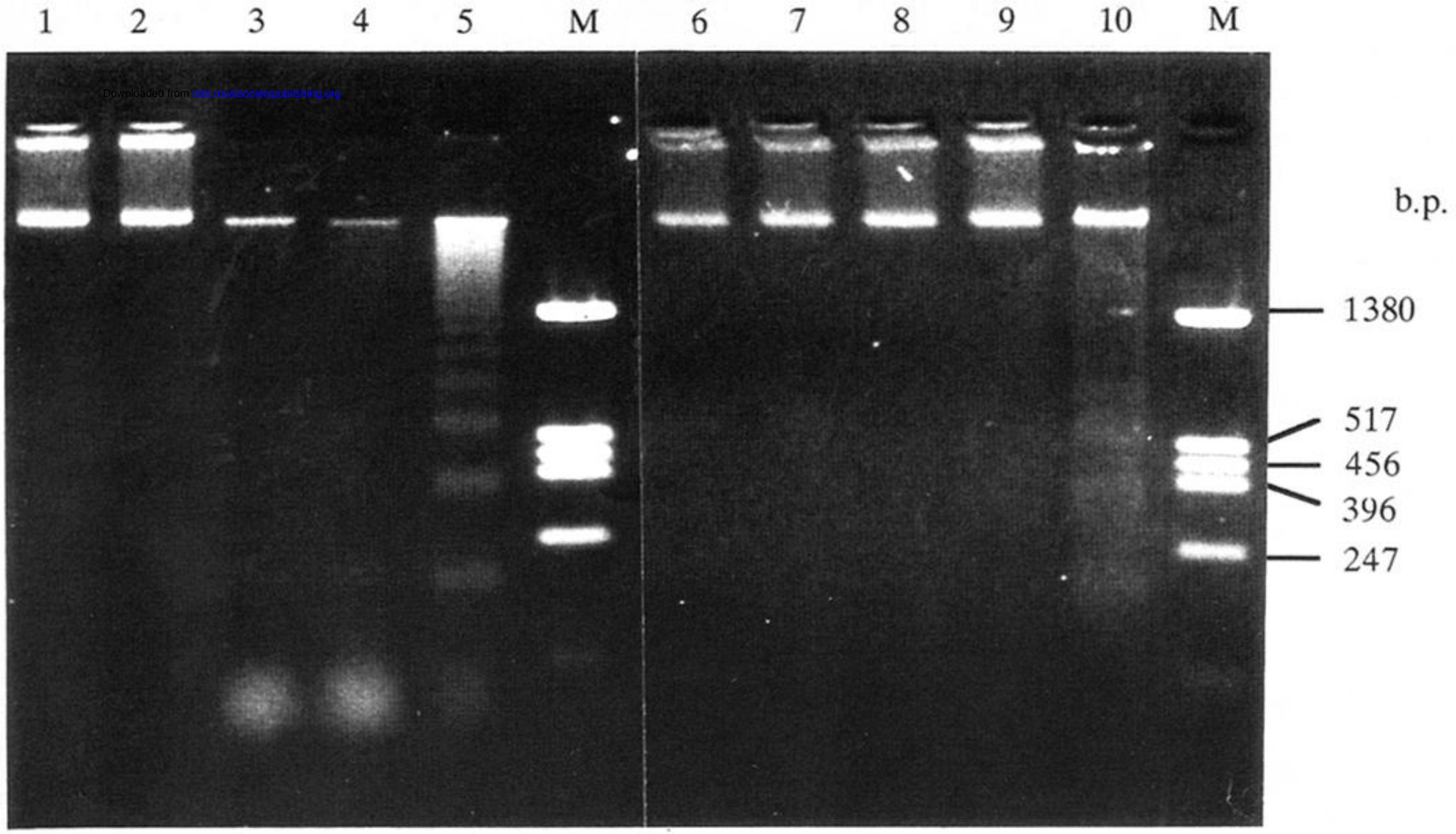

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