



Fig. 1 Nutritional requirement of the ILE401 line. *a*, Minimal RMOP (see Table 1 legend) medium; *b*, minimal medium with 200 mg l⁻¹ L-isoleucine; *c*, minimal medium with 100 mg l⁻¹ α-aminobutyric acid. Cultures were incubated for 3 weeks in the light (2,000 lx, 16 h).

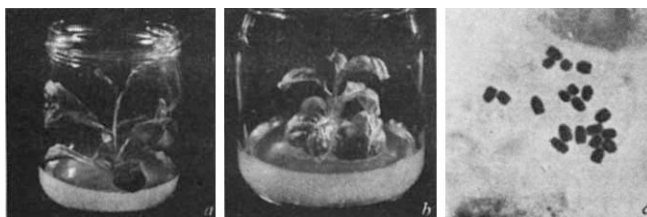


Fig. 2 Wild-type diploid *N. plumbaginifolia* (*a*) and ILE401 (*b*) plants. Metaphase chromosomes in root tips of an ILE401 auxotrophic plant are also shown (*c*).

Except for the first and last steps of their biosynthetic pathway, isoleucine and valine are synthesized by a common set of enzymes in microorganisms¹⁵ and in flowering plants¹⁶. As isoleucine alone is sufficient to restore normal growth of the ILE401 line, it may be defective in either threonine deaminase or transaminase B (Fig. 3). There was no detectable threonine deaminase activity in crude extracts of the ILE401 line, in contrast to the wild type, in which the specific activity was 1.0×10^{-3} μmol per min per mg protein, a value comparable with those found in other plant species^{17,18}. Transaminase B activity, however, was not affected in the mutant. Specific activities in the mutant and wild type were 5.2×10^{-4} and 3.9×10^{-4} μmol per min per mg protein, respectively. (Details of the assays of both enzymes are given in Fig. 3 legend.) The isoleucine requirement of the ILE401 line is therefore due to the absence of threonine deaminase activity. Normal functioning of the rest of the pathway was shown in growth tests in which isoleucine could be substituted with α-aminobutyric acid (Fig. 1), which is converted *in vivo* to α-ketobutyric acid¹⁹, the product of threonine deaminase. To avoid complications due to feedback inhibition by excess isoleucine²⁰, α-aminobutyric acid was used in the test, rather than α-ketobutyric acid, to provide a physiological concentration of α-ketobutyric acid (thus indirectly that of isoleucine).

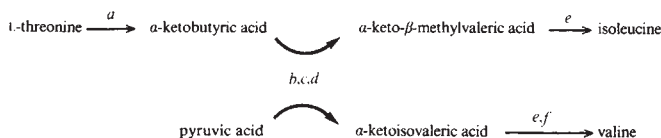


Fig. 3 The isoleucine-valine pathway. *a*, Threonine deaminase; *b*, acetohydroxy acid synthase; *c*, acetohydroxy acid isomeroreductase; *d*, dihydroxy acid dehydrase; *e*, transaminase B; *f*, transaminase C. Extracts for the enzyme assays were prepared by grinding 1 g leaf tissue in 3 ml buffer (50 mM KH₂PO₄, 20 mM 2-mercaptoethanol (2-ME), 2 mM EDTA, pH 8.0) and centrifuging the homogenate at 15,000g for 10 min. Threonine deaminase activity was measured at 37°C in the presence of 62.5 mM KH₂PO₄, 40 mM L-threonine, 5 mM 2-ME, 0.5 mM EDTA, pH 8.0. α-ketobutyric acid formed was determined by the direct method of Friedemann and Haugen²⁵. Transaminase B activity was measured at 37°C in the presence of 30 mM α-ketoglutaric acid, 25 mM L-isoleucine, 12.5 mM KH₂PO₄, 5 mM 2-ME, 0.5 mM EDTA and 0.1 mM pyridoxal phosphate, pH 8.0, using the reverse reaction. α-keto-β-methylvaleric acid formed was determined by the indirect method of Friedemann and Haugen²⁵.

The isoleucine requirement of the ILE401 line may be a result of a mutation or of epigenetic changes³. The low frequency of this phenotype, stability in culture for more than a year and expression at the plant level support the former³. Preliminary data on fusion with recessive mendelian pigment mutants indicate that isoleucine (and uracil) auxotrophies are recessive traits. A detailed genetic analysis of the ILE401 plants is in progress.

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Mouse IgG3 antibodies are highly protective against infection with *Streptococcus pneumoniae*

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Carbohydrate and protein antigens have been shown to elicit the bulk of their antibodies in mutually exclusive IgG subclasses in humans, mice, rats and horses¹⁻⁴. In the mouse, anti-protein antibodies are primarily of the IgG1 subclass, and anti-carbohydrate antibodies are primarily of the IgG3 subclass^{1,2}. We have now demonstrated that mouse IgG3 antibodies to the phosphocholine (PC) determinant of pneumococcal C-carbohydrate^{5,6} and to type 3 pneumococcal polysaccharide are highly protective against experimental type 3 pneumococcal infection. This is the first demonstration that antibodies of the IgG3 subclass can protect against bacterial infection.

Previously we have demonstrated that hybridoma IgM anti-PC antibody can protect mice from infection with type 3 *Streptococcus pneumoniae*⁷ strain WU2. Other data (ref. 8 and J. B. Robbins, personal communication) indicate that hybridoma anti-PC antibody can also protect against pneumococcal types 1, 4 and 6A. Much of the anti-PC antibody produced by inbred mice expresses the variable region idiotype marker, T-15 (ref. 9). Antibodies with this idiotype have virtually identical hyper-variable region sequences¹⁰, affinity for PC¹⁰ and specificity for PC analogues¹¹. We have used protection studies with T-15-

positive hybridoma antibodies of different isotypes¹¹ to compare the effects of differences in constant regions without having to consider differences in specificity.

CBA/J mice were infected intravenously (i.v.) with 10⁶ colony-forming units (CFU) of strain WU2 type 3 *S. pneumoniae*. This dose is 10 times the LD₅₀ (dose that kills 50% of infected mice). Passively administered IgG3 anti-PC antibody was almost 90-fold more protective against *S. pneumoniae* on a weight basis than IgM antibody of the same idiotype (Table 1). Similarly, IgG3 anti-type 3 antibody was almost 40 times more protective than IgM anti-type 3 antibody. Protection was not observed for IgA anti-PC antibody, IgM anti-salmonella antibody, or IgG3 anti-levan antibody. Anti-type 3 antibodies were ~6–15-fold more protective by weight than anti-PC antibodies. This was expected as the PC determinants, which form part of the cell wall⁶, should be largely buried beneath the type-specific capsule¹².

We repeated these studies with (CBA/N × DBA/2)F₁ mice, which express the X-linked immunodeficiency *xid*^{13,14}, make extremely low levels of natural anti-PC antibody^{7,15} and fail to produce anti-type 3 antibody when immunized¹³. These mice were infected with 150 CFU (~10 × LD₅₀) of *S. pneumoniae* strain WU2. IgG3 anti-PC antibodies protected *xid* mice ~10 times more effectively than IgM anti-PC antibodies, but IgG3 and IgM antibodies to the type 3 capsule showed roughly equal protective ability (Table 2). As with normal animals, anti-type 3 IgM was more effective than anti-PC IgM antibodies; however, IgG3 antibodies of the two specificities provided similar protection. The reasons for the different relative protective abilities of IgG3, IgM, anti-PC and anti-type 3 antibodies in normal and *xid* mice are unclear, but may be related to differences in immunoresponsive potential and levels of natural serum antibody of the two types of mice, or to the 4-log difference between the infectious doses used in the two studies.

Table 1 Ability of antibodies reactive with pneumococcal cell wall and capsule to protect CBA/J mice against infection with *S. pneumoniae* strain WU2

Hybridoma or myeloma*	Specificity	Idiotype†	Isotype†	PD ₅₀ (µg per injection)‡
59.6C5	PC	T-15	IgG3	0.6
PC-5-2	PC	T-15	IgM	55
T-15 and H-8	PC	T-15	IgA	>300
16.3	SSS-III	?	IgG3	0.1
CA3-1	SSS-III	Unique	IgM	4
CC4-6	SSS-III	Unique	IgA	>200
J606	Levan	J606	IgG3	>200
ST-1	<i>Salmonella typhimurium</i>	?	IgM	>200

Mice were infected i.v. with 10⁶ CFU of *S. pneumoniae*⁷, and protected with intraperitoneal (i.p.) injections of 0.1 ml of the indicated immunoglobulin in doses of 0.02, 0.2, 2.0, 20 or 200 µg (or 100 or 300 µg) 1 h before infection and 1 and 2 days after infection. Injection of diluent alone (0.1% fetal calf serum) was not protective. Antibody and diluent solutions were all filter-sterilized (0.22 µm) before injection. Over 80% of deaths occurred within 4 days of infection. The experiment was discontinued after 10 days.

* Myeloma antibodies T-15, H-8 and J606 and hybridoma protein 59.6C5 were isolated by affinity chromatography^{11,20,25}. Hybridoma proteins 16.3, CA3-1, CC4-6 (ref. 26), ST-1 (ref. 27) and PC-5-2 (a gift from John Kearney) were used as diluted ascites fluids. The amount of antibody in these fluids was quantitated by radioimmunoisotope assays²⁸. Comparable dilutions of normal mouse serum were not protective.

† Determination of the idiotypes and isotypes has been described previously^{9,11,26,28}; ? indicates unknown idiotype.

‡ PD₅₀, dose of passive antibody calculated²⁹ to protect 50% of the mice from fatal infection. At least six to eight mice were tested at each dose.

Table 2 Ability of antibodies reactive with pneumococcal cell wall and capsule to protect (CBA/N × DBA/2)F₁ male mice from *S. pneumoniae* strain WU2

Hybridoma or myeloma	Specificity	Idiotype	Isotype	PD ₅₀ (µg per injection)
59.6C5*	PC	T-15	IgG3	0.6
134.5B11*	PC	T-15	IgG3	0.7
PC-5-2	PC	T-15	IgM	6
22.1A4 ^c	PC	T-15	IgM	4
140.7C6.2*	PC	T-15	IgM	6
T-15 and H-8	PC	T-15	IgA	>300
16.3	SSS-III	?	IgG3	0.6
CA3-1	SSS-III	Unique	IgM	0.8
J606	Levan	J606	IgG3	>100
ST-1	<i>Salmonella typhimurium</i>	?	IgM	>200

The mice used here express the X-linked immunodeficiency (*xid*) defect of CBA/N mice, have no naturally occurring anti-PC antibody^{7,15} and are highly susceptible to pneumococcal infection. Mice were infected i.v. with 100 CFU of *S. pneumoniae* and protected as described in Table 1 legend.

* Anti-PC hybridoma antibodies¹¹ were produced and isolated as described in Table 1 legend.

The more efficient protection by IgG3 antibody in normal and *xid* mice is even more striking considering the fact that divalent IgG3 would be expected to have a much lower avidity than an IgM molecule for identical binding sites. IgM, which is only poorly recognized by Fc receptors^{16–18}, probably acts by opsonizing *S. pneumoniae*, using its highly efficient complement fixation¹⁹. The mechanism of IgG3-mediated killing is not so obvious. Although unaggregated IgG3 does not bind complement C1 (ref. 20), IgG3 anti-streptococcal group A carbohydrate antibody¹ can apparently lyse antigen-coated red blood cells in the presence of complement²¹. Thus the C3b receptor of phagocytes may play a part in IgG3-mediated bactericidal activity. Fc receptors for IgG3 (ref. 22) probably contribute to the anti-pneumococcal protection observed here, as they mediate phagocytosis of sheep red blood cells (SRBC) in the presence of IgG3 anti-SRBC antibody²².

The possibility that IgG3 may mediate bactericidal activity by an antibody-dependent cellular cytotoxicity is suggested by the fact that human natural killer cells can kill bacteria²³, and the recent demonstration that passive IgG3 anti-tumour antibody can protect against tumour growth far better than IgM antibody of the same specificity²⁴.

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The switch region associated with immunoglobulin C_μ genes is DNase I hypersensitive in T lymphocytes

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It is not known whether the antigen-specific receptor of T lymphocytes is encoded by conventional immunoglobulin genes. Several T-cell lines, as well as thymus cells, have been found to contain aberrant IgM mRNAs, but other T-cell lines are apparently negative¹⁻⁴. Rearrangement of *D* and *J* gene segments which code for portions of immunoglobulin heavy (H) chain variable (V) regions has been observed in some T cells, but no complete *V-D-J* rearrangements, as required for *H* gene expression in B cells, have been found⁵. To investigate the chromatin structure around the C_μ gene in T lymphocytes, we have probed the regions flanking the gene by mild digestion with DNase I. We report here that T-lymphocyte chromatin exhibits sites hypersensitive to DNase I in a region 5' of C_μ. In B cells, this region has been proposed to be responsible for switching to downstream C_H genes.

It has been observed in other systems that there are sites in chromatin which are hypersensitive to cleavage by DNase I⁶⁻¹⁰. The location of such sites can be related to the restriction map of a specific gene. It was postulated that the hypersensitivity occurs at DNA sites which may regulate gene activity in the vicinity⁶⁻¹⁰. To determine whether hypersensitive sites are associated with

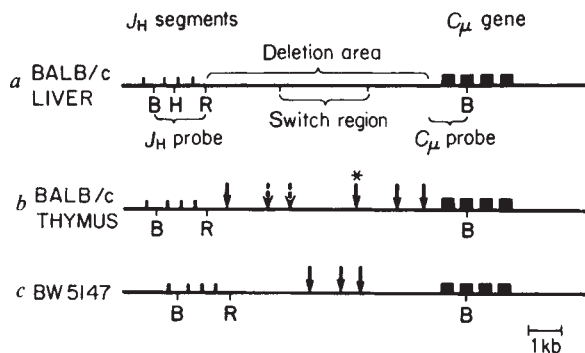


Fig. 1 Restriction maps of the region 5' of the C_μ gene (modified from Liu *et al.*¹⁶). Deletion area¹⁵⁻¹⁷ and switch region¹⁸⁻²² were reported for several plasmacytomas to be involved in the deletion of sequences 5' of C_μ and the switch to other C_H genes, respectively. Vertical arrows indicate DNase I-hypersensitive sites (the stippled arrows indicate sites in thymus visible with the J_H probe, but indistinct with the C_μ probe); *, site that was only visible with the C_μ but not with the J_H probe—this site seems also to be hypersensitive in purified DNA (see Fig. 3). The J_H probe is a BamHI-EcoRI fragment cloned by K. Marcu into pBR322 (ref. 15). The C_μ probe is a BamHI-EcoRI (artificial Eco site) fragment from a germ-line library clone subcloned into pBR322 (R. Near, E. Selsing and U.S., unpublished results).

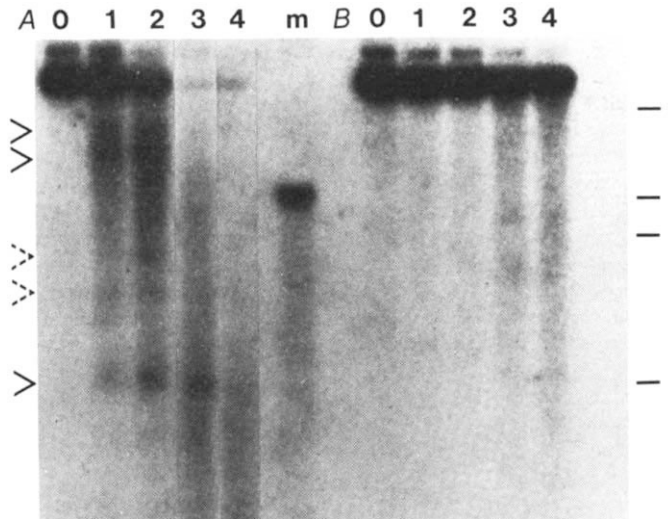


Fig. 2 Southern blots of thymus (A) and liver (B) DNAs from untreated nuclei (track 0) and nuclei treated with DNase I (tracks 1-4) to reduce gradually the size of the DNA to an average of 15-20 kb (track 4)¹². The DNAs were prepared and digested with BamHI¹³. Southern blots²⁸ were produced and hybridized with a J_H probe (see Fig. 1a). Sizing markers are indicated along the right-hand side of the gel—λ phage DNA cut with *Ava*I (8.5, 6.1, 4.8 and 2.2 kb). m, Radiolabelled sizing marker due to the hybridization of the plasmid probe with the 4.6-kb phage λ DNA fragment. Subfragments flanked by a BamHI site and a DNase I-hypersensitive site are indicated by arrowheads. The major hybridization band is the J_H-C_μ gene; the faint fragments above it are due to incomplete digestion by BamHI.

the C_μ gene in T lymphocytes, nuclei from thymus cells (>99% T lymphocytes¹¹) and liver cells (as a control) were prepared and mildly treated with DNase I¹². The range of DNase I concentrations used has been shown to digest active immunoglobulin genes without affecting inactive genes and the bulk of the DNA¹². DNA was extracted from these nuclei and analysed by restriction enzyme digestion together with Southern filter hybridization to map DNase I-hypersensitive sites⁸. A restriction map of the C_μ gene, indicating the enzyme sites used in the analyses, is shown in Fig. 1a.

Liver cells were analysed to provide controls representing a tissue in which immunoglobulin genes are not expressed^{12,13}. When liver DNA, extracted from untreated nuclei, was digested with BamHI and hybridized with a particular J_H probe (see Fig. 1a) a single restriction fragment of 9.8 kilobases (kb) was observed (Fig. 2B, track 0). DNAs extracted from liver nuclei treated with increasing amounts of DNase I showed the same BamHI restriction fragment (Fig. 2B, tracks 1-4). No additional bands were observed, indicating that there are no DNase I-hypersensitive sites in this region of the liver genome.

Thymus DNA, extracted from untreated nuclei, and hybridized with the J_H probe also showed the 9.8-kb BamHI restriction fragment (Fig. 2A, track 0) seen with liver DNA. This supports the conclusion that in most thymus cells no DNA rearrangement has occurred in the region between J_H and the C_μ gene. In contrast to the results obtained with liver cells, however, DNase I treatment of thymus nuclei resulted in the appearance of new fragments (indicated by arrows in Fig. 2A). These subfragments must be flanked by a BamHI site at one end and a DNase I-hypersensitive site at the other. As the J_H probe represents the 5' end of the 9.8-kb J_H-C_μ BamHI fragment (Fig. 1a), the length of the subfragments produced by DNase I digestion indicates the distance between the BamHI site within the J_H cluster and the DNase I-hypersensitive sites (Fig. 1b) thus allowing localization of the hypersensitive sites in the J_H-C_μ map.

We have confirmed the location of DNase I-hypersensitive sites in thymus chromatin by analysis with a C_μ probe (see