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Separation of multiple genes controlling the T-cell proliferative response to IL-2 and anti-CD3 using recombinant congenic strains

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Abstract T lymphocytes of the strain BALB/cHeA exhibit a low proliferative response to IL-2 and a high response to the anti-CD3 monoclonal antibodies, while the strain STS/A lymphocyte response to these stimuli is the opposite. We analyzed the genetic basis of this strain difference, using a novel genetic tool: the recombinant congenic strains (RCS). Twenty BALB/c-c-STS/Dem (CcS/Dem) RCS were used, each containing a different random set of approximately 12.5% of the genes from STS and the remainder from BALB/c. Consequently, the genes participating in the multigenic control of a phenotypic difference between BALB/c and STS become separated into different CcS strains where they can be studied individually. The strain distribution patterns of the proliferative responses to IL-2 and anti-CD3 in the CcS strains are different, showing that different genes are involved. The large differences between individual CcS strains in response to IL-2 or anti-CD3 indicate that both reactions are controlled by a limited number of genes with a relatively large effect. The high proliferative response to IL-2 is a dominant characteristic. It is not caused by a larger major cell subset size, nor by a higher level of IL-2R expression. The response to anti-CD3 is known to be controlled by polymorphism in Fcγ receptor 2 (*Fcgr2*) and the CcS strains carrying the low responder *Fcgr2* allele indeed responded weakly. However, as these strains do respond to immobilized anti-CD3, while the STS strain does not, and as some CcS strains with the BALB/c allele of *Fcgr2* are also low responders, additional gene(s) of the STS strain strongly depress the anti-CD3 response. In a backcross between the high responder and the low responder strains CcS-9 and CcS-11, one of these unknown genes was mapped to the chromosome 10 near *D10Mit14*. The CcS mouse strains which carry the *STS* alleles of genes controlling the proliferative response to IL-2 and anti-CD3 allow the future mapping, cloning, and functional analysis of these genes and the study of their biological effects in vivo.

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Introduction

The immune response is a complex process which can be broken down into several stages: recognition of antigen, signal transmission, and mounting of the effector reactions. The antigen-induced activation of T lymphocytes is a complex process (Jenkins and Johnson 1993; Wegener et al. 1992) resulting in changes in the expression of at least 100 genes (Ullman et al. 1990). T cells can be activated to produce cytokines and cytokine receptors which drive proliferation and differentiation, as well as cell surface and intracellular molecules involved in effector functions. One of the major events in T-lymphocyte activation is the induction of the expression of IL-2 and its receptor, which allows autocrine stimulation of proliferation (Miyajima et al. 1992; Minami et al. 1993).

Mouse strains differ considerably in their ability to respond to antigenic challenge. Some of these differences are caused by a single gene, others are polygenically regulated. Mouse strains with impairment in the immune response have proven invaluable tools for addressing a broad spectrum of immunological problems (Todd et al. 1991; Wicker et al. 1992; Peterson et al. 1992; Malek et al. 1989; Lipoldová et al. 1992). However, in cases where several nonlinked loci are responsible for the strain differences, the mapping of these genes may be difficult, even using the recombinant inbred strains (Klein and Taylor 1985; Roberts et al. 1993).

Therefore, a special tool has been developed for genetic analysis of multigenically-controlled complex biological processes: the recombinant congenic strains [(RCS) (Demant and Hart 1986; Demant et al. 1989; Demant 1992)]. A series of RCS comprises about 20 homozygous strains, all produced by inbreeding from the second backcross genera-

tion of two inbred strains, one of which serves as a parental background strain and the other as a parental donor strain. Each RC strain of the series contains a different, random, set of about 12.5% genes from the parental donor strain and about 87.5% genes of the parental background strain. The donor gene sets of individual RCS overlap in approximately 1.5% of genes. The RCS system has been used to analyze the multigenic control of susceptibility to colon and lung tumors (Moen et al. 1991; Moen et al. 1992; Fijneman et al. 1994) and susceptibility to radiation-induced apoptosis in thymus (Mori and co-workers, in press). In this paper we describe the application of the RCS for analysis of the genes controlling lymphocyte proliferation induced by IL-2 and anti-CD3, and the mapping of the genetic control of proliferative response after anti-CD3 stimulation.

Materials and methods

Mice

Mice of strains BALB/cHeA (BALB/c), STS/A (STS) and recombinant congenic strains of the BALB-c-STS/Dem (CcS/Dem) series, 8 to 22 weeks old, were from the breeding colony of P. Demant. The genetic composition of these RCS has been described in detail (Groot et al. 1992; Groot and co-workers, in press). The experiments described in this paper were carried out over the course of several years and repeated with mice from several separate shipments with essentially the same result. When used for these experiments, the strains of the CcS/Dem series were in the generation 17-30 of inbreeding, and hence highly homozygous (Groot and co-workers, in press).

IL-2

Human recombinant IL-2 (sp. act. 18×10^6 IE/mg) was obtained from EuroCetus Benelux B. V. (Amsterdam).

Antibodies

The monoclonal antibody (mAb) KT3 (Tomonari 1988) recognizing the mouse CD3 complex (Huang and Crispe 1993) was obtained from K. Tomonari (MRC Clinical Research Centre, Harrow, U. K.). Hybridoma PC 61 (Lowenthal et al. 1985) which served as a source of mAb anti-α chain of the IL-2R was provided by M. Nabholz (Swiss Institute for Experimental Cancer Research, Epalinges s/Lausanne, Switzerland). Anti-CD4 monoclonal antibodies H129-19 and anti-CD8 mAb 53-6.7 have been described (Pierres et al. 1984; Ledbetter and Herzenberg 1979).

Lymphocyte proliferation assay

Spleen cells (10^5 cells per well) were stimulated with IL-2 or anti-CD3 mAb at several concentrations in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-ME, 25 mM HEPES buffer, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). All reagents were from Sigma (St. Louis, MO). Anti-CD3 stimulation was tested with both the soluble and immobilized antibody. Anti-CD3 was immobilized by incubation in phosphate buffered saline (PBS) at 37 °C for 1 h, followed by 1 h incubation at 4 °C, and removal of the remaining soluble antibody by 3 × washing with PBS. In some experiments spleen cells were grown in the serum-free medium AIM V (Gibco BRL, Vienna, Austria). [^{3}H]thymidine [(Institute for Research, Development and Utilization of Radioisotopes, Prague, Czech Republic) (0.5 μCi/well)] was added into the cultures for the last 6 h of the 72 h incubation period. Medium RPMI 1640 supplemented with FCS from Sigma yielded the lowest background value and all data presented in the paper were obtained with this FCS. However, no differences in the strain distribution pattern of proliferation after IL-2 or anti-CD3 stimulation were seen, using FCS from two other sources: Bioveta (Ivanovice na Hané, Czech Republic) and SEBAK GmbH (Aidenbach, Germany), or the serum-free medium AIM V (data not shown).

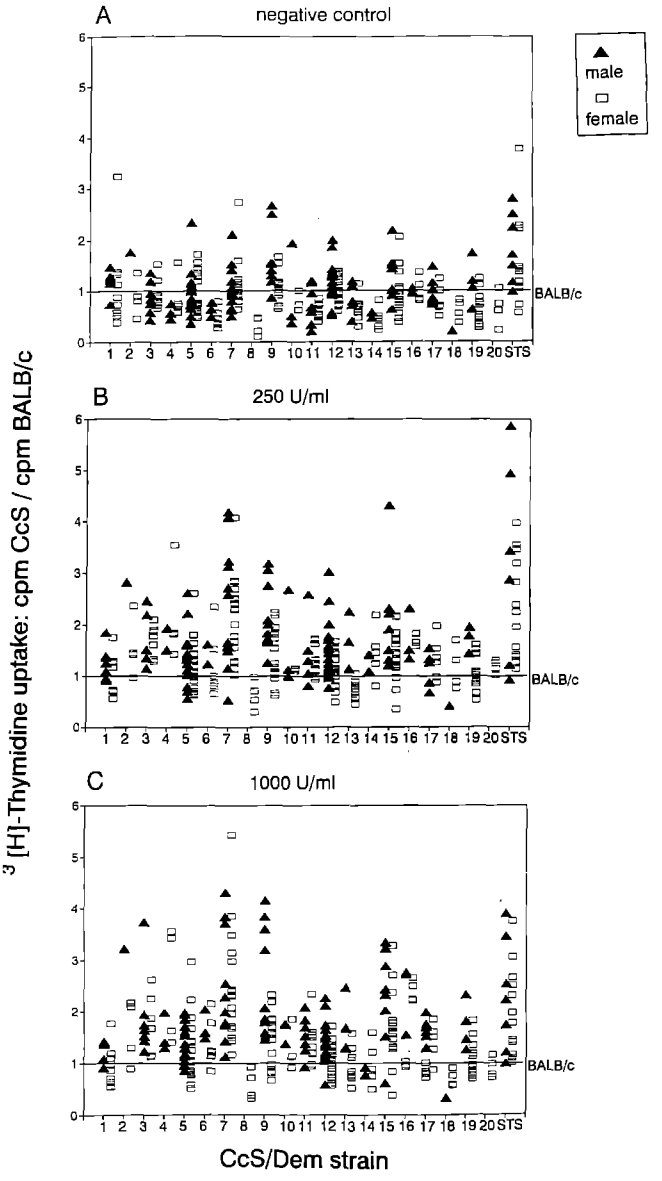

Fig. 1A–C Comparison of proliferative response to IL-2 in CcS/Dem series with respect to BALB/cHeA. Scattergram of **A** unstimulated spleen cell cultures and of **B** proliferative response to IL-2 in concentration 250 units/ml and **C** 1000 units/ml. Data summarize 30 experiments; only those in which strain BALB/c was used are included. Each value represents the mean of triplicate cultures in counts per min of CcS/Dem strain divided by the value obtained in the same experiment by the background strain BALB/cHeA. The mean value of [^{3}H]thymidine incorporation by BALB/c was **A** 904, **B** 5481, and **C** 15911 cpm. The following strain abbreviations are used: 1–20, CcS – strain 1–20

Table 1 Percentage increase in proliferative response to IL-2 with respect to BALB/cHeA. Percentage of increase in [^{3}H]thymidine incorporation in various CcS strains and hybrids as compared with BALB/c. The 95% confidence interval is shown in brackets. The mean values of BALB/c [^{3}H]thymidine uptake was 904, 4605, 5481, 8542, 15911, and 27628 cpm when spleen cell cultures were stimulated by 0, 125, 250, 500, 1000, and 2000 units/ml IL-2, respectively

Strain	IL-2 doses											
	0		125 units/ml		250 units/ml		500 units/ml		1000 units/ml		2000 units/ml	
1	**5**	(–8/22)	**1**	(–12/17)	**18**	(2/36)	**12**	(–2/30)	**9**	(–5/26)	**7**	(–7/24)
2	**8**	(–12/34)	**32**	(6/64)	**43**	(15/78)	**49**	(20/86)	**47**	(18/83)	**40**	(13/74)
3	**2**	(–13/21)	**36**	(14/61)	**56**	(32/86)	**63**	(37/94)	**71**	(44/103)	**61**	(35/91)
4	**–3**	(–23/20)	**58**	(26/99)	**83**	(46/130)	**88**	(50/137)	**93**	(54/143)	**65**	(31/108)
5	**–4**	(–17/10)	**–2**	(–15/12)	**7**	(–6/23)	**12**	(–2/29)	**15**	(0/32)	**19**	(3/37)
6	**–42**	(–52/–30)	**–2**	(–19/17)	**9**	(–9/32)	**38**	(14/68)	**55**	(28/88)	**66**	(37/100)
7	**11**	(–2/27)	**105**	(79/135)	**124**	(95/156)	**140**	(110/175)	**140**	(109/175)	**116**	(89/148)
8	**–65**	(–72/–55)	**–39**	(–52/–22)	**–31**	(–46/–12)	**–35**	(–49/–17)	**–31**	(–46/–12)	**–26**	(–42/–6)
9	**46**	(27/69)	**47**	(28/70)	**71**	(48/97)	**75**	(52/102)	**84**	(60/113)	**68**	(46/95)
10	**–19**	(–35/0)	**6**	(–15/32)	**12**	(–9/40)	**8**	(–12/35)	**31**	(5/64)	**37**	(10/72)
11	**–34**	(–45/–22)	**10**	(–7/32)	**20**	(0/43)	**22**	(2/46)	**33**	(11/59)	**34**	(12/61)
12	**5**	(–9/22)	**0**	(–13/17)	**20**	(3/39)	**10**	(–5/28)	**16**	(0/35)	**16**	(0/35)
13	**–32**	(–44/–19)	**–12**	(–26/5)	**–5**	(–21/13)	**0**	(–16/20)	**20**	(0/44)	**36**	(14/64)
14	**–37**	(–48/–23)	**21**	(0/48)	**24**	(1/51)	**0**	(–18/21)	**–7**	(–24/13)	**–21**	(–36/–4)
15	**4**	(–10/19)	**36**	(18/57)	**52**	(31/75)	**78**	(54/105)	**98**	(72/129)	**95**	(69/124)
16	**9**	(–43/112)	**21**	(–37/134)	**42**	(–26/176)	**30**	(–32/152)	**46**	(–24/184)	**43**	(–25/177)
17	**–4**	(–27/25)	**7**	(–18/40)	**17**	(–10/54)	**34**	(3/77)	**37**	(5/80)	**57**	(20/106)
18	**–37**	(–50/–22)	**–7**	(–25/16)	**–6**	(–25/16)	**–11**	(–29/9)	**–20**	(–36/0)	**–23**	(–38/–3)
19	**–28**	(–39/–15)	**–2**	(–17/14)	**5**	(–10/24)	**16**	(–1/36)	**20**	(2/42)	**14**	(–2/34)
20	**–38**	(–50/–22)	**4**	(–16/30)	**7**	(–13/34)	**–9**	(–27/13)	**–4**	(23/18)	**–4**	(–23/19)
STS	**101**	(67/142)	**212**	(160/276)	**215**	(162/279)	**187**	(13/245)	**148**	(106/198)	**108**	(73/150)
F_1 (BALB × 7)	**–11**	(–29/10)	**62**	(29/103)	**68**	(34/111)	**73**	(38/117)	**83**	(46/130)	**74**	(38/118)
F_1 (7 × BALB)	**–12**	(–31/10)	**64**	(0/108	**75**	(38/122)	**80**	(42/128)	**104**	(61/158)	**76**	(39/123)

IL-2 production assay

Spleen cells from individually tested mice were incubated in 96-well tissue culture plates (Nunc) in a concentration of 0.5×10^6 cells/ml in complete RPMI 1640 medium alone or in the presence of 1.5 µg/ml anti-CD3 mAb. After 4 h, 24 h, 48 h, or 72 h of incubation, the supernatants were harvested and tested for the ability to support the growth of IL-2-dependent T-cell line CTLL-2 (Gillis et al. 1978). In brief, 100 µl of CTLL-2 cell suspension (5×10^4 cells/ml) were incubated with equal volumes of serially diluted supernatants, and cell proliferation was determined by adding 0.5 µCi of [^{3}H]thymidine/well for the last 6 h of the 24 h incubation period.

Preparation of cell subpopulations

Highly purified T- or B-cell populations were obtained by combining separation techniques of nylon wool adherence (Julius et al. 1973) and panning (Wysocki and Sato 1978) as described previously [Holáň et al. 1985). The purity of the resulting cell population was controlled by FACScan analysis, using anti-Ig (Sigma) and and anti-CD3 (Tomonari 1988) for B and T cells, respectively. Within the limits of the method's sensitivity, the populations appeared to be pure. In a biological assay for the presence of antigen presenting cells (APC) the CD3$^+$ cells prepared by this method did not respond by proliferation to concanavalin A or anti-CD3.

Immunofluorescence analysis

Fluorescence staining was performed on ice in 50 µl containing 6×10^5 cells and a predetermined amount of primary antibody in PBS containing 2.5% mouse serum and 0.1% NaN_3. Primary antibodies were labeled with fluorescein isothiocyanate FITC. In some experiments a fluoresceinated secondary antibody was used. Staining with a secondary antibody was performed in a similar manner after the cells were washed. All analyses were performed by using a FACSTAR cell sorter (Becton Dickinson, Mountain View, CA).

Genotyping of simple sequence length polymorphisms (SSLP) by PCR

PCR genotyping was performed as described (Groot et al. 1992), using the primers from Research Genetics (Huntsville, FL). Briefly, amplification of DNA was performed with an MJ Research Thermal Cycler PTC 100 Model 96 (MJ Research, Watertown, MA) in a volume of 10 µl with 20 ng DNA, 0.23 µM of forward primer γ[^{32}P] ATP end-labeled with T4 polynucleotide kinase (New England Biolabs, Schwalbach, Germany), 0.23 µM of reverse primer, 200 µM of each dNTP and 0.04 units *Taq* polymerase (GIBCO, Grand Island, NY) in a 96-well U-bottom microtiter plate (FALCON, Oxford, UK) with 1 cycle of 3 min at 94 °C and 30 cycles of 30 sec at 94 °C, 1 min at 55 °C and 1 min at 72 °C and 1 cycle of 1 min at 55 °C, 3 min at 72 °C, and 1 min at 23 °C. Each PCR product was mixed with 30 µl of loading buffer and electrophoresed in 6% acrylamide gels for 2–2.5 h at 50 W. Gels were wrapped in Saran Wrap (Dow Chemical Co., Midland, MI) and exposed to X-ray film X-format AR (Kodak, Weesp, The Netherlands) for 1.5–24 hours at –70 °C.

Statistical analysis

An analysis of variance with repeated measurements (with the Compound Symmetry model for the within-animal covariance matrix) was performed, using the logarithm of counts as dependent variable; strains, experiments, and gender as between animal factors; IL-2 or anti-CD3 dose as within-animal factor; and age as covariate. Calculations were done, using the statistical package BMDP-PC90 (BMDP Statistical Software Manual): program 2V for significance tests (the Greenhouse-Geisser correction was used for the calculation of the *P*-values where appropriate), program 5V for estimation of the effects (using the REML method), and program DM for the plots. Evaluation of linkage was performed after logarithmic transformation of counts to obtain normal distribution by analysis of variance (Advanced ANOVA, NCSS), using the genotype and sex as fixed factors and the experiment as random factor.

Results

Proliferative response to IL-2

Pilot experiments revealed that the strain BALB/cHeA exhibits a low proliferative response to IL-2, whereas the strain STS/A shows a high response to this stimulus. The responses of BALB/c, STS, and the twenty CcS/Dem RC strains were tested at five different concentrations of IL-2. A total of 506 male and female mice, 8 to 22 weeks old, were used. The results are given in Table 1 and Figure 1. In this Table and Figure the data observed in the RC strains and STS are related to the mean value observed in the BALB/c mice in each experiment. The mean c. p. m. values with BALB/c lymphocytes were 904 (0 units/ml IL-2), 4605 (125 units/ml), 5481 (250 units/ml), 8542 (500 units/ml), 15911 (1000 units/ml), and 27628 (2000 units/ml). Without stimulation, the strains CcS-9 and STS have higher levels of [^{3}H]thymidine incorporation compared with BALB/c, while strains CcS-6, -8, -11, -13, -14, -18, -19, and -20 have lower levels ($P < 0.0001$).

For all IL-2 doses the strains CcS-2, -3, -4, -7, -9, and -15 have increased levels of stimulation with respect to the strain BALB/c ($P < 0.0001$). The highest response was observed in RC strains CcS-7, CcS-9, and CcS-15. The IL-2 dose-effect curves of strains CcS-6, -15, and -17, and the donor strain STS/A markedly and significantly deviate in shape from the general pattern observed in other strains. STS/A has a higher response to the lower concentrations of IL-2, whereas CcS-6, -15, and -17 are relatively weakly stimulated in the lower dose region but respond better in the higher dose region (see Table 1). Comparison of the response of males and females revealed no significant difference in overall level ($P = 0.49$), but the shape of the IL-2 dose-effect curve differed between males and females ($P < 0.0001$), females being more stimulated in the lower dose region. No effect of age within the period of 8 to 22 weeks on the proliferative response was found ($P = 0.74$).

The strain differences appear to hold over a wide range of IL-2 concentrations as well as cell concentrations: in tests of the strains BALB/c, STS, CcS-9, and CcS-11 in several concentrations ranging from 0.125×10^6 to 2×10^6 cells/ml and IL-2 ranging from 125 units/ml to 2000 units/ml, the strain STS was the high responder, the strain BALB/c low, and the strains CcS-9 and CcS-11 were intermediate (data not shown).

If the differences in IL-2 response between BALB/c and STS were controlled by an additive effect of a very large number of nonlinked loci, each CcS strain would likely receive at least some of them from the donor strain, and hence most or all CcS strains would exhibit a similar, intermediate response. However, the large differences in proliferative response between individual CcS strains and the number of high- and low-responder strains suggest that a limited number of genes with a major effect is involved in the controlling of IL-2-stimulated proliferation. The highly responding strain CcS-7 was selected for further investigation. The proliferative response of F_1 hybrids between BALB/c and CcS-7 to IL-2 exhibited increased levels of stimulation with respect to BALB/c (Table 1; Fig. 2), indicating that the allele causing a high proliferative response to IL-2 in this strain is dominant.

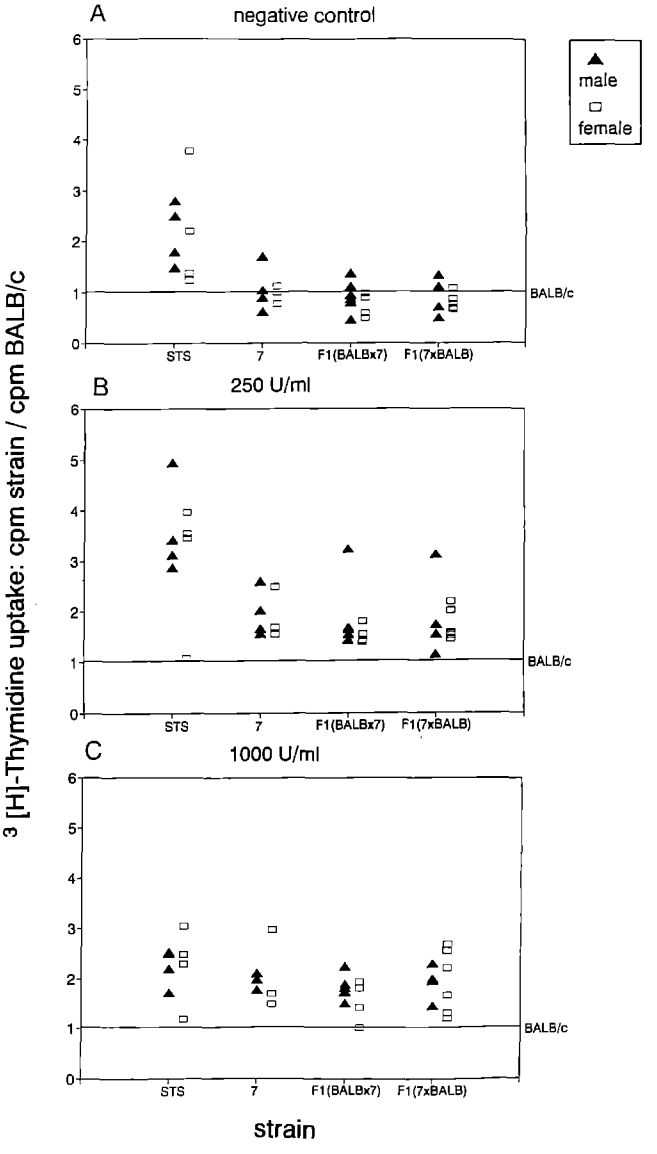

Fig. 2A–C Comparison of proliferative response to IL-2 in F_1 hybrids between BALB/c and CcS-7 and their parents. Scattergram of **A** unstimulated spleen cell cultures and of **B** proliferative response to IL-2 in concentration 250 units/ml, and **C** 1000 units/ml. Each value represents the mean of triplicate cultures in counts per min divided by the value obtained in the same experiment by the background strain BALB/c. The mean value of [^{3}H]thymidine incorporation by BALB/c was **A** 655, **B** 4339, **C** 15543 cpm

Analysis of cell subpopulations by flow cytometry

To establish whether the higher response to IL-2 reflects the size of one of the major T-cell subpopulations or the preexisting expression of the IL-2 receptor, freshly obtained spleen cells from high (STS, CcS-4, -7)- and low

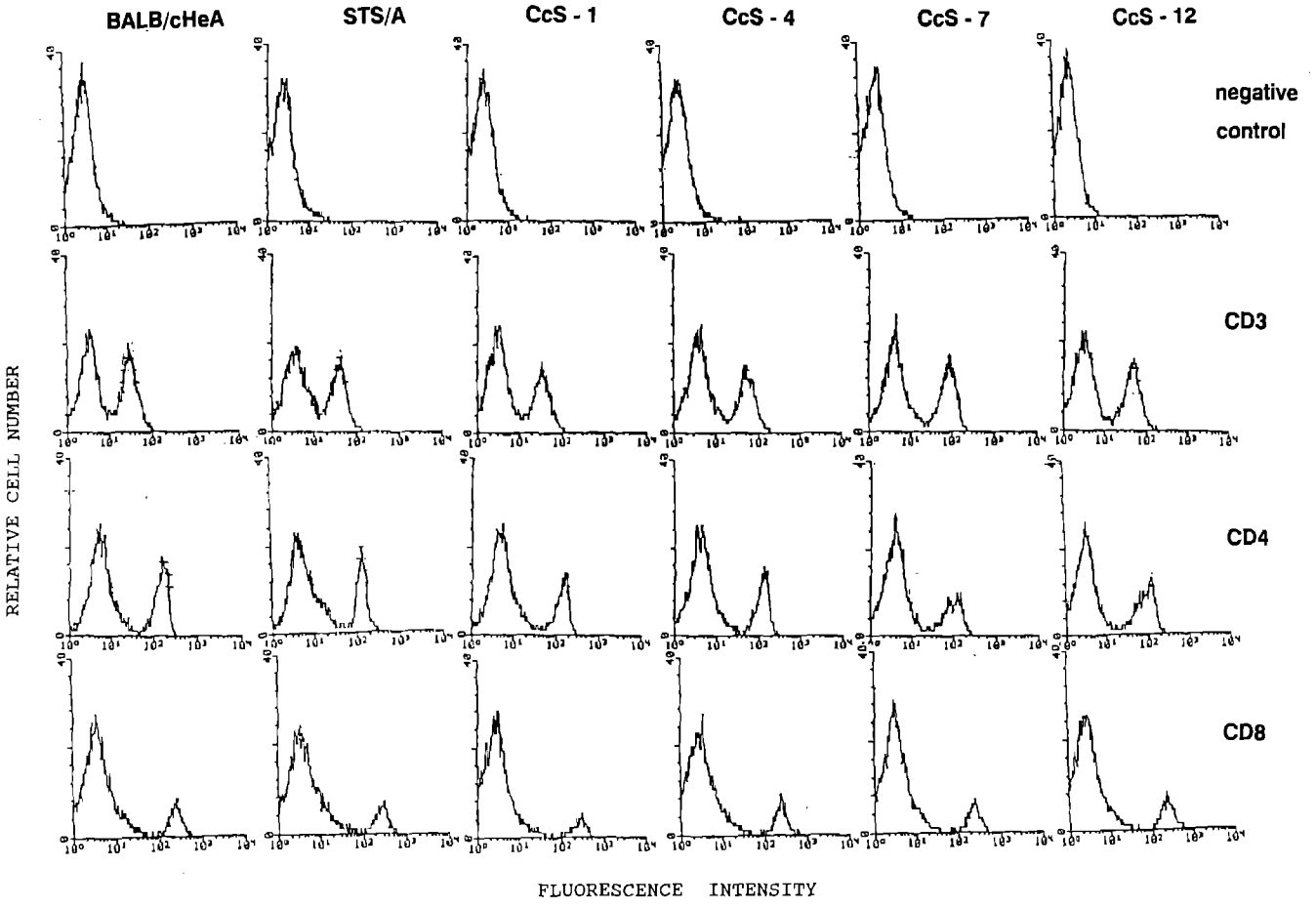

Fig. 3 Flow cytometric analysis of freshly obtained mouse spleen cells. Cells from strains representing low (BALB/c, CcS-1, -12) and high (STS/A, CcS-4, -7) responders to IL-2 were stained with FITC-conjugated antibodies as described in Materials and methods. Cell number versus fluorescence intensity is shown

(BALB/c, CcS-1, -12)-responder strains were analyzed by FACS. No significant differences with respect to the frequency of $CD3^+$, $CD4^+$, and $CD8^+$ subsets were found (Fig. 3). No $CD25^+$ cells were detected before IL-2 stimulation, and after stimulation the expression of CD25 was very low, with no differences between high- and low-responder strains (data not shown).

Response to IL-2 by purified cell subpopulations

The proliferative response to IL-2 by unseparated spleen cells, enriched T cells or B cells was compared. Figure 4 documents that the strain differences observed between the proliferative response of the total spleen cell population reflect the differences between the response of $CD3^+$ populations.

Strain differences in proliferative response to soluble anti-CD3 mAb

The proliferative response to anti-CD3 mAb was analyzed in 234 males and females (Table 2; Fig. 5). The epitope recognized by this mAb is present in all strains of CcS/Dem series, and the frequency of $CD3^+$ cells is similar both in intermediate- and high-responder strains BALB/cHeA, CcS-1, and CcS-4 and low responders STS/A, CcS-7, and CcS-12 (Fig. 3). The strain differences in response to anti-CD3 stimulation have been reproducible in a number of separate experiments for a period of more than 2 years. The higher response of the strains BALB/c, CcS-9, and CcS-15, as compared with the low-responder strain STS was detectable in cell concentrations ranging from 0.25×10^6 to 1×10^6 (data not shown).

The absence of a functional CD32 (*Fcgr2*) receptor in humans has been reported to be associated with the inability to proliferate after anti-CD3 stimulation (Tax et al. 1983). The background strain BALB/c shows a higher response to anti-CD3 mAb, whereas donor strain STS exhibits low response; this finding corresponds with the presence of functional CD32 antigen in BALB/cHeA and its absence in STS/A (Hibbs et al. 1985). Therefore, we tested whether this was the only factor determining the genetic differences in anti-CD3 response. Genetic typing indicated that the STS/A allele of *Fcgr2* producing nonfunctional CD32 is present also in CcS-7, -11, and -12 (Groot and co-workers, in press). These three CcS strains were indeed very low responders, as expected. However, the 17 CcS strains with the functional *Fcgr2* allele still exhibit large differences in

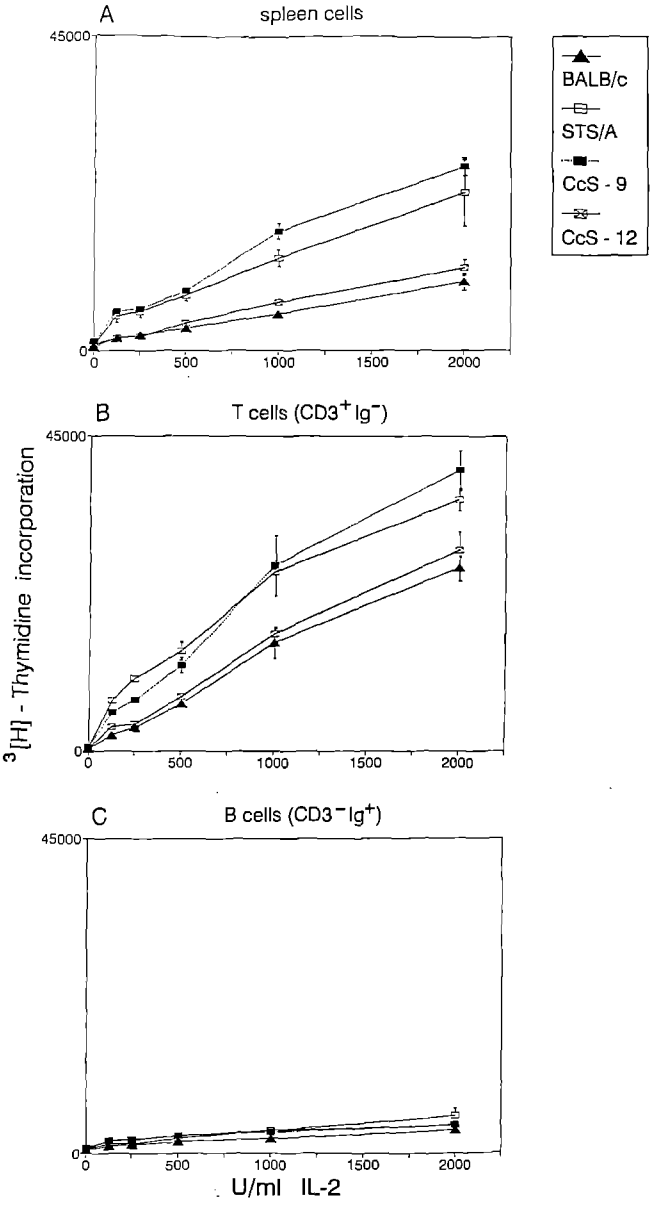

Fig. 4A–C Comparison of proliferative response to **A** IL-2 by spleen cells, **B** enriched T cells ($CD3^+$, Ig^-) and **C** B cells ($CD3^-$, Ig^+). T and B cells were obtained as described in Materials and methods. Results represent arithmetic means ± SD of triplicate determinations in one representative experiment. Seven independent experiments gave similar results

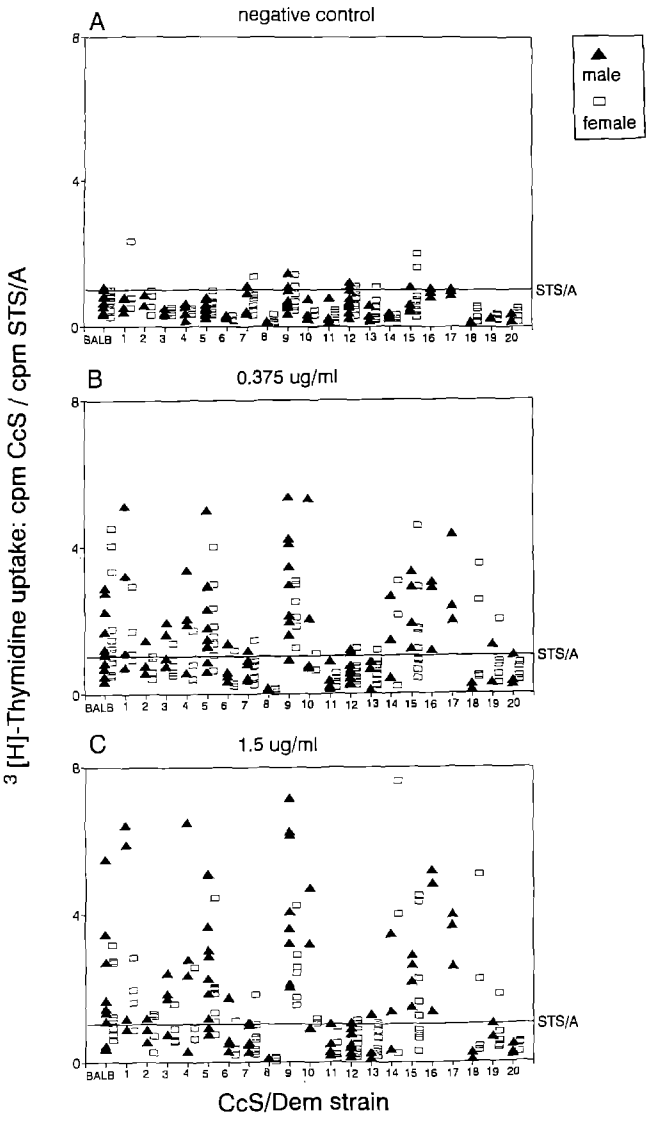

Fig. 5A–C Comparison of the proliferative response to anti-CD3 mAb in the CcS/Dem series with respect to STS/A. **A** Scattergram of [^{3}H]thymidine incorporation of nonstimulated spleen cell cultures and **B** the proliferative response to anti-CD3 mAb in concentrations of 0.375 μg/ml and **C** 1.5 μg/ml, representing data from all experiments in which strain STS/A was used. Each value represents the mean of triplicate cultures in cpm of CcS/Dem RCS divided by the value obtained for the response of donor strain STS/A in the same experiment. The mean value of [^{3}H]thymidine incorporation by STS/A was **A** 1498, **B** 2023, and **C** 2643 cpm. Abbreviations used in this Figure: 1–20, CcS – strains 1–20

their ability to respond by proliferation ($P < 0.0001$). CcS-4, -5, and -9, exhibit a higher proliferative response than BALB/cHeA, CcS-6, -8, -13, -18, and -20, show decreased levels of stimulation with respect to BALB/cHeA for all anti-CD3 doses, and CcS-8, CcS-18, and CcS-20 (as well as CcS-11 and CsS-12) are even lower responders than the donor strain STS/A. These data demonstrate that besides *Fcgr2* several other genes having a large effect are involved in the control of the anti-CD3-stimulated response.

Analysis of variance indicated that the CD3-dose-effect curves of strains CcS-1, -4, -5, -9, and -10, and STS/A markedly and significantly deviate in shape from the pattern observed in the other strains ($P < 0.0001$; Table 2). CcS-1, -5, -9, and -10 show a better response in comparison with BALB/c in higher concentrations of anti-CD3, CcS-4 exhibits a higher increase in proliferative response in an intermediate-rather than in a high-dose region, whereas the donor strain STS, which is a low responder, responds relatively better to low doses of anti-CD3 than in the high-dose region.

Table 2 Percentage increase in proliferative response to anti-CD3 mAb with respect to BALB/cHeA. Percentage of increase in [3H]thymidine incorporation in various CcS strains as compared with BALB/c. The 95% confidence interval is shown in brackets. The mean values of BALB/c [3H]thymidine uptake was 1157, 1875, 3389, 5030, 6966, and 10235 cpm when spleen cell cultures were stimulated by 0, 0.092, 0.375, 1.5, 6, and 24 μg/ml anti-CD3 mAb, respectively

Strain	Anti-CD3-concentration					
	0	0.92 μg/ml	0.375 μg/ml	1.5 μg/ml	6 μg/ml	24 μg/ml
1	**10** (–28/69)	**26** (–17/94)	**35** (–12/107)	**58** (3/143)	**63** (5/150)	**94** (26/199)
2	**6** (–33/68)	**–4** (–39/51)	**–14** (–45/36)	**–24** (–52/19)	**–3** (–39/52)	**–5** (–40/49)
3	**–4** (–35/41)	**9** (–26/62)	**28** (–13/90)	**34** (–9/99)	**35** (–8/101)	**37** (–7/103)
4	**7** (–34/74)	**20** (–26/96)	**78** (9/191)	**100** (22/227)	**155** (56/318)	**98** (21/224)
5	**–18** (–40/12)	**11** (–19/53)	**65** (20/128)	**72** (25/138)	**104** (47/181)	**120** (59/203)
6	**–47** (–65/–19)	**–49** (–66/–22)	**–44** (–63/–15)	**–48** (–66/–21)	**–50** (–67/–24)	**–40** (–61/–9)
7	**–6** (–35/37)	**–25** (–48/9)	**–41** (–60/–14)	**–53** (–68/–31)	**–61** (–73/–44)	**–61** (–73/–43)
8	**–65** (–79/–41)	**–76** (–86/–59)	**–83** (–90/–72)	**–90** (–94/–83)	**–91** (–94/–85)	**–92** (–95/–87)
9	**18** (–15/64)	**47** (5/105)	**136** (69/229)	**199** (115/318)	**284** (176/436)	**278** (171/427)
10	**–30** (–56/13)	**–12** (–45/41)	**49** (–7/142)	**59** (–1/158)	**99** (23/223)	**118** (35/253)
11	**–36** (–56/–5)	**–47** (–64/–22)	**–53** (–68/–30)	**–57** (–71/–37)	**–68** (–78/–52)	**–71** (–81/–58)
12	**–2** (–28/34)	**–34** (–52/–9)	**–50** (–64/–32)	**–64** (–74/–51)	**–71** (–79/–61)	**–76** (–82/–67)
13	**–40** (–59/–13)	**–46** (–62/–21)	**–49** (–65/–26)	**–54** (–68/–34)	**–56** (–70/–37)	**–65** (–76/–50)
14	**–51** (–69/–23)	**–22** (–50/22)	**–6** (–40/47)	**5** (–33/65)	**6** (–32/67)	**8** (–31/69)
15	**–0** (–30/41)	**2** (–28/46)	**13** (–20/62)	**3** (–27/47)	**–4** (–33/36)	**17** (–17/68)
16	**14** (–32/94)	**–10** (–47/53)	**–13** (–49/63)	**–3** (–43/63)	**3** (–38/76)	**22** (–27/108)
17	**–5** (–44/61)	**1** (–40/72)	**26** (–25/114)	**28** (–24/119)	**14** (–32/95)	**3** (–38/76)
18	**–54** (–72/–25)	**–57** (–74/–30)	**–46** (–67/–128)	**–60** (–75/–35)	**–56** (–73/–28)	**–59** (–74/–33)
19	**–45** (–64/–16)	**–39** (–60/–7)	**–33** (–56/0)	**–36** (–58/–3)	**–36** (–58/–3)	**–29** (–54/7)
20	**–45** (–66/–12)	**–47** (–67/–15)	**–46** (–66/–13)	**–66** (–78/–45)	**–67** (–79/–47)	**–71** (–82/–54)
STS	**42** (3/95)	**1** (–26/38)	**–23** (–44/4)	**–28** (–48/–2)	**–51** (–64/–34)	**–63** (–73/–50)

Proliferative response to immobilized anti-CD3

The strain STS is a low responder to both soluble and immobilized anti-CD3. However, the strains CcS-7, CcS-11, and CcS-12, which share with STS the nonfunctional *Fcgr2* allele and are low responders to soluble anti-CD3, are high responders to immobilized anti-CD3 (Fig. 6). This shows that the *Fcgr2* allele is not the only genetic factor responsible for the low responsiveness of the STS strain.

Low proliferative response to anti-CD3 is accompanied by decreased synthesis of IL-2

Supernatants obtained after anti-CD3 stimulation of spleen cells of low (STS, CcS-11)- and high (BALB/c, CcS-5, CcS-9)-responder strains were tested for the presence of IL-2. The levels of IL-2 correlated with the proliferative response of the strains tested: spleen cells of STS did not produce detectable IL-2, the supernatants of CcS-11 contained either very low or undetectable levels, and BALB/c, CcS-5, and CcS-9 produced intermediate or high amounts of IL-2 (Fig. 7).

Mapping the genes controlling anti-CD3 proliferative response

As the first step of identification of genes controlling the differences in anti-CD3-proliferative response, we performed a mapping experiment in a cross between a high-responder RC strain (CcS-9) and a low-responder RC strain (CcS-11). The two strains differ at the *Fcgr2* locus, but probably also at additional loci controlling the anti-CD3 response. A backcross CcS-11 × (CcS-11 × CcS-9)F_1 was prepared. The individual backcross mice were tested for the proliferative response at different concentrations of anti-CD3, and their DNA was typed for the genetic markers at which the two CcS strains differ. A large difference was found between the heterozygotes and homozygotes at the *Fcgr2* locus ($P < 0.00001$), the latter being low responders. In addition, considerable phenotypic heterogeneity was observed between mice heterozygous at the *Fcgr2* locus. The effects of the genotype at other markers located on 12 different chromosomes in this group which comprised 82 mice are summarized in Table 3. The multilocus analysis of variance indicates a significant effect of the genotype at the *D10Mit14* locus on chromosome 10 ($P = 0.002$) and suggested the possible involvement of two other regions, on chromosomes 16 and 17. These latter two, however, do not reach the required significance level, and hence need to be tested in additional crosses.

Discussion

Lymphocyte activation has been extensively studied and is known to involve coordinated transcriptional activation of a large array of genes (Ullman et al. 1990). T-cell-proliferative reactions to stimulation by IL-2 and anti-CD3 simulate different stages of the antigen-induced T-cell activation (Ullman et al. 1990), and they apparently employ signalling pathways with at least partly distinct elements (Down-

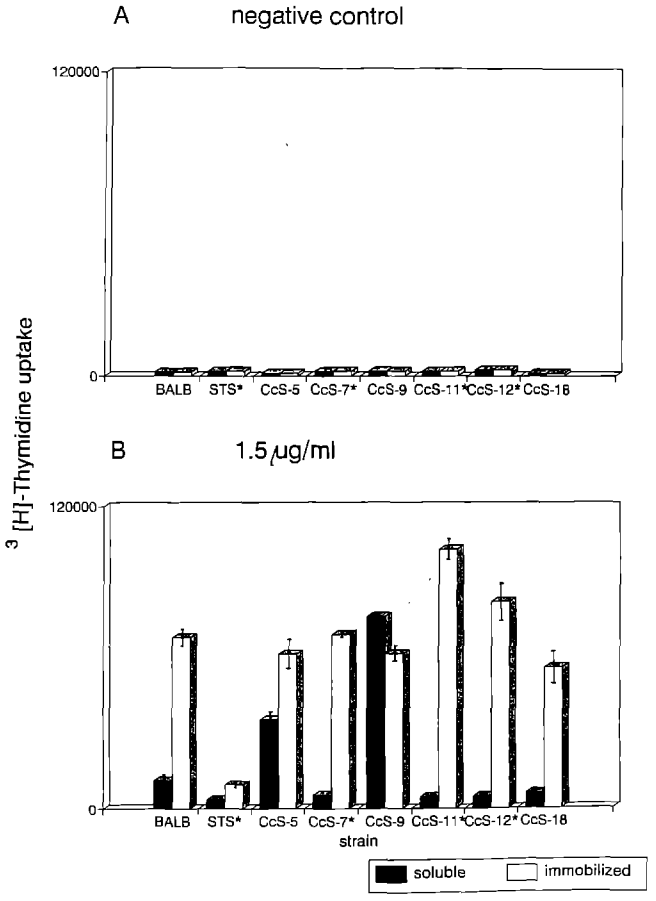

Fig. 6A, B Comparison of proliferative response to soluble and immobilized anti-CD3. **A** Non-stimulated cells. **B** 1.5 µg/ml anti-CD3 was used for stimulation in solution, as well as for immobilization. Spleen cell proliferation was tested as described in Materials and methods. An *asterisk* indicates the strains with the *STS* allele of *Fcgr2*. Seven independent experiments gave similar results

ward et al. 1992). We used the RCS system to analyze two different T-lymphocyte reactions – the proliferative response to IL-2 and anti-CD3. Our data show large genetic differences in the intensity of these reactions and indicate that they are influenced by a different, limited set of genes with relatively large effects.

We decided to study the IL-2 response because of its important role in driving lymphocyte proliferation, and because it is a relatively simple lymphocyte reaction. The experiments with purified cell subpopulations (Fig. 4) show that the strain differences in the IL-2-induced proliferative response reflect the proliferation of CD3+ lymphocytes. These genetic differences in the IL-2-induced proliferative response may be the result of several factors: differences in the number of IL-2 receptor molecules, efficacy of the signal transduction process, rate of autocrine IL-2 synthesis, the capacity of T cells to become costimulated by the B-cell activation antigen B7 (Schwartz 1992; Linsley et al. 1991 a, b), or by differences in size of the responding cell subpopulations. These possibilities are not mutually exclusive; on the contrary, our data show that several nonlinked genes with large effects determine the difference in the IL-2 proliferative response between BALB/cHeA and STS/A, and it is not likely that all these genes operate through the same mechanism. Our data suggest that at least some of the mechanisms listed above are not likely. Analysis by flow cytometry indicated that the size of CD3+, CD4+, and CD8+ subpopulations is similar in both high- and low-responder strains (Fig. 3). CD25+ cells have not been detected by this method before stimulation, and there were no detectable differences between high- and low-responder strains in the density of CD25-staining after stimulation. The level of IL-2 mRNA in spleen cell cultures stimulated by IL-2 for different periods was slightly higher in STS than in BALB/cHeA, but we did not observe any significant difference in expression of IL-2 mRNA between BALB/c and the high

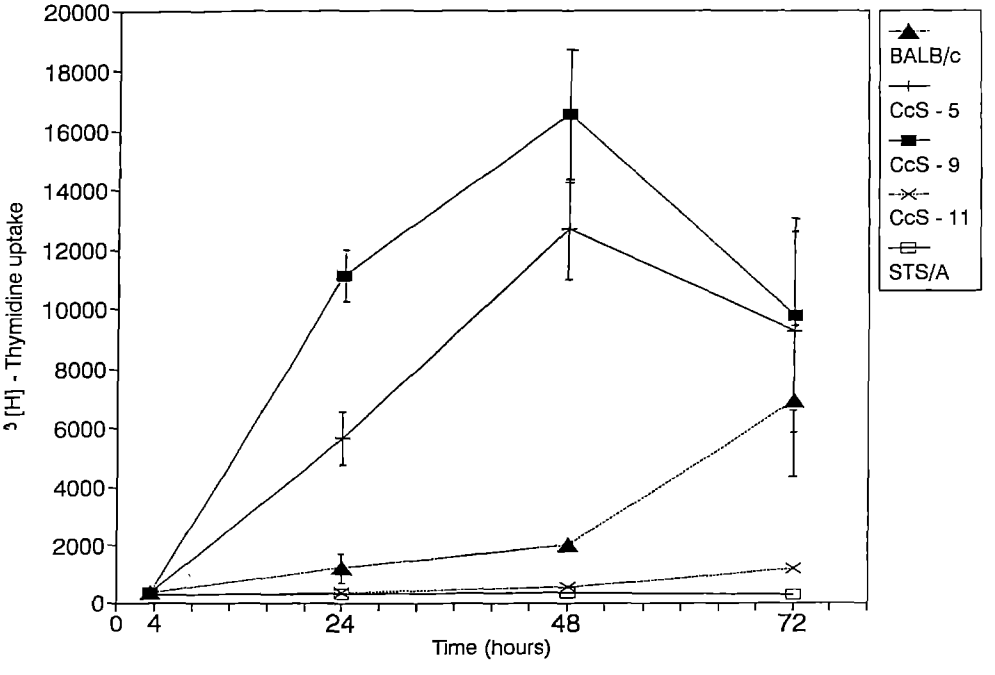

Fig. 7 Synthesis of IL-2 after stimulation with anti-CD3 mAb. Supernatants from spleen cell cultures of high (BALB/cHeA, CcS-5, -9) and low (STS/A, CcS-11) responders stimulated by 1.5 µg/ml anti-CD3 mAb were harvested at different time periods and tested for their ability to support the growth of the IL-2-dependent T-cell line CTLL-2 as described in Materials and methods. The dilution of supernatants was 1:2. The data shown in this figure represent six separate experiments. *Vertical bars* represent SD of the mean of two determinations. Incorporation of 20000 cpm represents approximately 300 pg of IL-2. In supernatants from cultured unstimulated spleen cells no IL-2 was detected by this method. The mean value of CTLL cells cultured in medium only was 370 cpm

Table 3 Linkage analysis of anti-CD3 response in *Fcgr2* heterozygous CcS-11 × (CcS-9 × CcS-1)F_1 backcross mice

Chromosome	Locus	*P*-value
3	*D3Mit18*	N. S.
4	*D4Mit7*	N. S.
	D4Mit23	N. S.
5	*D5Mit24*	N. S.
6	*D6Mit42*	N. S.
7	*D7Mit7*	N. S.
	D7Mit14	N. S.
	D7Mit54	N. S.
	D7Nds2	N. S.
	D7Nds5	N. S.
9	*D9Mit15*	N. S.
10	*D10Mit14*	**0.002**
	D10Mit46	N. S.
11	*D11Mit8*	N. S.
	D11Mit26	N. S.
	D11Nds9	N. S.
	D11Nds18	N. S.
12	*D12Mit37*	N. S.
16	*D16Mit7*	0.050
	D16Mit34	N. S.
17	*D17Mit38*	0.052
	D17Mit42	N. S.
	D17Mit120	N. S.
19	*D19Mit16*	N. S.

responders CcS-7, -9, -15, and (BALB × CcS-7)F_1 (data not shown). This indicates that the interstrain differences in the IL-2 response are not due to a pre-existing antigenic stimulation in some CcS/Dem strains but not in others. IL-2 stimulates activated T cells, mediates augmentation of natural killer cells, and induces the growth of B cells and some cell subsets of nonlymphoid origin (Minami et al. 1993). The CcS/Dem strains that carry the *STS* alleles of these genes on BALB/c background may be suitable tools for defining and analyzing the mechanisms of their action in vitro and in vivo. Polymorphism in the proliferative response to anti-CD3 mAb has been described both in humans (Tax et al. 1983) and in mice (Hibbs et al. 1985). It has been attributed to the effect of polymorphism of the Fcγ receptor 2 (*Fcgr2*) on the function of monocytes/macrophages (Hibbs et al. 1985), and spleen monocytes were also required for anti-CD3-induced proliferation in our experiments (data not shown). The parental strain STS carrying the *Fcgr2* allele producing a nonfunctional CD32 molecule and three other CcS/Dem strains that received the *Fcgr2* allele from STS are low responders, as expected. Nevertheless, there must be at least one other gene with a considerable influence on this response as well, because the strains CcS/Dem-6, -8, -13, -18, and -20 carrying the BALB/c *Fcgr2* allele are low responders. Evidence for an additional gene controlling the anti-CD3 response is provided by experiments with immobilized antibody. STS is a low responder, but the strains CcS-7, CcS-11, and CcS-12, sharing the same *Fcgr2* allele, are low responders to soluble anti-CD3, but high responders to immobilized anti-CD3. Moreover, several CcS/Dem strains are even higher responders than BALB/c, indicating that they received from the low-responder parent STS a gene enhancing the anti-CD3-stimulated response.

The proliferation induced by anti-CD3 mAb differed significantly in Th1 and Th2 cell lines (Gajewski et al. 1990; Wang et al. 1993) and the regulatory role of the CD4 molecule (Tamura et al. 1990) and cytokines (de Waal Malefyt et al. 1993) in this response was described. As the strain distribution pattern (Fig. 5; Table 2) and dose-response curves (Table 2) also indicate that anti-CD3-induced proliferation is controlled by more than one gene, the low anti-CD3 proliferative response in different CcS/Dem strains may be the result of different mechanisms. The strain distribution patterns of IL-2- and anti-CD3-induced proliferative responses are different (Figs. 1, 5; Tables 1, 2), indicating that distinct sets of controlling genes are involved. However, some parts of the signalling pathway of these reactions could overlap (Downward et al. 1992). In addition, stimulation by anti-CD3 in our experiments is followed by synthesis of IL-2, high anti-CD3-responders being higher producers of this cytokine than low-responder strains (Fig. 7). Genes which determine the IL-2-response could thus influence the anti-CD3 response as well. Indeed, high anti-CD3-responders CcS-4, -9 also show a high proliferative response to IL-2, but the strains CcS-1, -5, -10, and BALB/c exhibit a high proliferative response to anti-CD3, whereas their response to IL-2 is low (Tables 1, 2). It is likely that anti-CD3-stimulated T cells of these strains produce an amount of IL-2 high enough to induce a high proliferative response in spleen cell cultures that exhibit a low response to direct stimulation by IL-2. The mapping of the genes controlling the IL-2 and anti-CD3 response will ultimately reveal whether some of them influence both responses.

The strain differences observed in this study cannot be simply explained by differences in the microbiological status of the strains: strains located in the same room exhibited differences in their response to IL-2 and anti-CD3, which were consistent for a period of more than 2 years. A remarkable feature of the present data is the extent of genetic variation influencing the activation of T cells between two inbred strains, BALB/c and STS. Although neither of these strains is immunodeficient, our results show that a number of genes influencing the two T-cell activation mechanisms tested exhibit allelic variation with considerable functional effects. So far, very little is known about the extent of the polymorphism of the genes controlling quantitative variation in the immune response in humans or in experimental animals, but our data suggest that it is likely to be extensive. The impact of this type of variation on various immunological reactions (resistance to infection, autoimmunity, capacity to reject or accept allogeneic transplant, inducibility of tolerance) is not known. However, identification of the genes involved in the mouse will make it possible to search for the homologous genes in the human.

The separation of these genes in individual mouse RC strains makes it possible to study their functions and will enable us to map them to specific chromosomal segments. The CcS/Dem RCS series is suitable for this study, because the two parental inbred strains, BALB/c and STS, differ considerably in both responses. In addition, the CcS/Dem RC strains were genetically characterized for more than 320 markers differing between the strains BALB/c and STS (Groot and co-workers, in press). Thus, in each CcS/Dem strain, we were able to define with considerable precision those segments of its genome which originate from BALB/c and those inherited from STS/A. This facilitates the future mapping of those genes responsible for the observed phenotypic differences, using the strategy previously applied to analysis of susceptibility to colon tumors (Moen et al. 1992) and to radiation-induced apoptosis in thymus (Mori and co-workers, in press). In a mapping experiment involving a backcross between the high- and low-responder RC strains to anti-CD3 stimulation CcS-9 and CcS-11, a significant association between the *Fcgr2* genotype and responsiveness was found, indicating consistency of the mapping with the earlier data (Tax et al. 1983; Hibbs et al. 1985). In addition, a novel locus influencing the proliferative response to the CD3 antibody was mapped to a region on chromosome 10, close to *D10Mit14*. As several of the low-responder RC strains do not carry an *STS* allele at the *Fcgr2* or the *D10Mit14* locus (CcS-2, -6, -8, -18, -20), they are likely to carry low-responder alleles at other loci controlling the anti-CD3 response. These loci can be mapped in appropriate crosses of these RC strains. These data show that the sensitivity of the RCS approach combined with the multilocus mapping analysis allows the identification of multiple chromosomal regions involved in the control of the anti-CD3 response, and probably can also be applied to other immunological phenomena.

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