substitution from Val to Ala [nomenclature 122 (50), T → C, Val → Ala]; 207 (135), C → T, 215 (143), C → T, Ala → Val; 224 (152), T → C, Ile → Thr; 231 (159), G → A; 247 (175), C → T, Pro → Ser; 251 (179), A → T, Glu → Val; 261 (189), T → C; 301 (229), A → G, Met → Val; 336 (264), T → G, Asn → Lys; 423 (348), A → G; 448 (373), A → C; 466 (391), C → A, Gln → Lys; 479 (404), A → G, Asp → Gly; 484 (409), G → A, Glu → Lys; 487 (412), G → A, Val → Ile; 542 (467), C → T, Thr → Met; 718 (631), C → T, His → Tyr; 834 (747), G → A; 857 (770), G → A, Arg → His; 864 (777), T → C; 865 (778), G → A, Glu → Lys; 953 (866), C → T, Ser → Leu; and 955 (868), T → G, Leu → Val. In NOD, there is an insertion of 3 nt, AAG, at the 3′-end of exon 3, an insertion of 12 nt, CTTTC-CCTTTAG, at the 3′-end of exon 4, and a deletion of 4 nt, GAGA, after position 996 (910 to 913).
20. M. F. Seldin, T. H. Roderick, B. Paigen, *Mammalian Genome* 1, S1 (1991).
21. J. C. Unkeless, E. Scigliano, V. H. Freedman, *Annu. Rev. Immunol.* **6**, 251 (1988).
22. H. Kikutani and S. Makino, *Adv. Immunol.* **51**, 285 (1992).
23. J. V. Ravetch and J.-P. Kinet, *Annu. Rev. Immunol.* **9**, 457 (1991).
24. K.-P. Lee, K. Amano, J.-W. Yoon, *Diabetes* **37**, 989 (1988); P. Hutchings *et al.*, *Nature* **348**, 639 (1990).
25. L. Shen, P. M. Guyre, C. L. Anderson, M. W. Fanger, *J. Immunol.* **137**, 3378 (1986).
26. H. Nakajima *et al.*, *Immunol. Lett.* **12**, 91 (1986).
27. We designed primers to amplify the transmembrane/cytoplasmic tail (T/C) coding region of *Fcgr1* from genomic DNA of the ICR mouse strain (5′-GTCCCCAGTCATCAGCTCCTG-3′ and 5′-AGAGTTGCATGCCATGGTCCC-3′). PCR reactions (100-μl) were done with biotinylated and nonbiotinylated primers in a Perkin-Elmer Thermocycler for 32 cycles comprising 1.5 min at 94°C, 1 min at 60°C, and 0.5 min at 72°C. Single-stranded DNA was prepared with Dynabeads M-280 Streptavidin. Chain termination DNA sequencing was done with Sequenase Version 2.0.
28. M. Sensi, P. Pozzilli, A. N. Gorsuch, G. F. Bottazzo, A. G. Cudworth, *Diabetologia* **20**, 106 (1981).
29. J. M. H. Debets, J. G. Van de Winkel, J. L. Cueppens, I. E. Dieteren, W. A. Buurman, *J. Immunol.* **144**, 1304 (1990).
30. C. O. Jacob, S. Aiso, S. A. Michie, H. O. McDevitt, H. Acha-Orbea, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 968 (1990).
31. T. Mandrup-Poulsen, K. Bendtzen, C. A. Dinarello, J. Nerup, *J. Immunol.* **139**, 4077 (1987).
32. Y. Ohta, T. P. Stossel, J. H. Hartwig, *Cell* **67**, 275 (1991).
33. D. Baker *et al.*, *J. Neuroimmunol.* **28**, 261 (1990).
34. S. Kataoka *et al.*, *Diabetes* **32**, 247 (1983).
35. The following strains were checked for the presence of the deletion in the cytoplasmic region of *Fcgr1* by PCR (*10*): A/J, AKR/J, B6.PL, BALB/c, high antibody responder Biozzi (AB/H), low antibody responder Biozzi (AB/L), C3H/HeJ, C57BL/6J, C57BL/10SnJ, C57Br/cdJ, C57L/J, Cast/Ei, CBA/J, DBA/2J, ICR, MRL/MpJ-+/+, MRL/MpJ-*lpr/lpr*, NOD, NON, NZB/B1NJ, NZW/lacJ, PL/J, *Mus spretus*/CRC, and SWR/J (*33, 36, 37*). A 184-bp product was obtained from NOD and AB/H, and a 188-bp product was obtained from the other strains.
36. G. Biozzi, D. Mouton, C. Stiffel, Y. Bouthillier, *Adv. Immunol.* **36**, 189 (1984).
37. M. F. Lyon and A. G. Searle, Eds., *Genetic Variants and Strains of the Laboratory Mouse* (Oxford Univ. Press, Oxford, 1989), ed. 2.
38. B10 mice were from the Jackson Laboratory (Bar Harbor, ME), and NOD mice were from Taconic Farm, Germantown, NY. CFA was prepared with heat-killed *Mycobacterium tuberculosis* (strain H37Ra) at a final concentration of 1.0 mg/ml, and an emulsion was made with an equal volume of sterile Dulbecco's phosphate-buffered saline. All mice were injected with 50 μl of CFA in each of the hind feet and boosted with 0.1 ml of CFA in the peritoneal cavity (IP) 14 days later. Flow cytometric analysis was performed 7 to 14 days after the IP injection on either a FACScan or FACStarPLUS (Becton Dickinson). MAb to Mac-1 (Hybritech, San Diego, CA) was used. FITC-conjugated antibody to rat κ (clone MARK-1) and MAb to IgG2a (clone H106.112.52) (AMAC, Westbrook, ME) were used. Phycoerythrin (PE)–conjugated and FITC-conjugated antibody to $IgG2a^b$ (clone 5.7), FITC-IgG2a (clone SF1-1.1), and FITC-IgG3 (IgG3, κ; Pharmingen, San Diego, CA) were used. All antibodies were titrated and used at saturation. All incubations and washes were performed at 4°C. We added propidium iodide to exclude dead cells from the analysis.
39. W. Dietrich *et al.*, *Genetics* **131**, 423 (1992).
40. Linkage was tested in 106 diabetic animals and 89 nondiabetic progeny with insulitis from the (NOD × B10.*H-2*^{g7})F_1 × NOD backcross and the reciprocal cross, which have been described (*1*). Insulitis was scored as described (*1*). Pair-wise recombination fractions and SEs are given. Genotype frequency differences were evaluated by χ^2 test of independence (1 df).
41. First backcross mice were bred with NOD and B6.PL–*Thy-1*^{a} and B10.*H-2*^{g7} mice according to the breeding schemes (NOD × B6.PL–*Thy-1*^{a})F_1 × B6.PL–*Thy-1*^{a}, and B10.*H-2*^{g7} × (B10.*H-2*^{g7} × NOD)F_1 and scored for the IgG2a phenotype (*38*). Discordant animals either expressed the phenotype and were B10.*H-2*^{g7} or B6.PL–*Thy-1*^{a} homozygous or did not express the phenotype and were heterozygous at the marker locus. Marker loci *D3Nds6* (*Il-2*), *D3Nds1*, *D3Nds7* (*Cacy*), *D3Nds8* (*Tshb*), and *D3Nds9* (*Adh-1*) have been described (*1, 9, 15*). Previous marker loci names are given in parentheses. *D3Mit22* has been described (*39*). We ordered marker loci by minimizing double recombinants. Pair-wise recombination fractions and SEs are given. Statistical analysis was by χ^2 test of independence (1 df).
42. NOD.*D3Nds7 D3Nds8* congenic mice were derived from NOD.*D3Nds6 D3Nds8* congenic mice (*11*). The *D3Nds7-D3Nds8* region was made homozygous by selective intercrossing. After six backcross generations, it is expected that 0.8% (~13 cM) of the genome will be of donor origin. The genome of NOD.*D3Nds7 D3Nds8* mice was fingerprinted with microsatellites. With this approach, the number of backcrosses required to produce informative congenic strains is reduced. The following microsatellites were used: *D1Nds1, D1Mit5, D2Mit11, D2Mit14, D2Mit22, D3Nds6, D3Mit6, D3Nds1, D3Mit22, D3Nds7, D3Nds11, D3Nds8, D4Nds3, D4Nds2, Lck, D4Nds13, D5Mit11, D5Nds2, D6Mit14, D7Nds2, D9Mit20, D9Nds3, D10Mit2, D10Nds1, D11Nds14, D11Nds16, D11Nds1, D11Nds17, D11Nds15, D12Mit2, D14Nds4,* and *D19Mit1* (*15, 39*). Only *D3Nds7, Fcgr1,* and *D3Nds8* were of B6.PL–*Thy-1*^{a} origin.
43. We thank J. Ravetch for discussion, J. Love for assistance, I. Jackson and J. Dorin for preparation of cosmid 6.11, I. Brown and D. Wraith for materials from the high and low antibody responder Biozzi mice, S. Makino and M. Hattori for genomic DNA from ICR mice, and E. Lander and W. Dietrich for primer sequences provided before publication. Supported by the Wellcome Trust, the Medical Research Council as part of the Human Genome Mapping Project, the Juvenile Diabetes Foundation, and the British Diabetic Association.

2 November 1992; accepted 25 January 1993

TECHNICAL COMMENTS

Immuno-PCR with a Commercially Available Avidin System

Antibody-based detection (*1*) and in vitro DNA amplification (*2*) together constitute a system that unifies two revolutionary methods of modern biology. Amplification of an antibody-born DNA label greatly enhances the signal. A method of immuno–polymerase chain reaction (immuno-PCR) was recently described by T. Sano *et al.* (*3*). We have developed an antigen detection system, similar to an enzyme-linked immunosorbent assay (ELISA), that uses a biotinylated antibody and an avidin-biotinylated DNA complex. We use commercially available avidin instead of the streptavidin–protein A–chimera, as proposed by Sano and Cantor (4). Each system has its advantages and drawbacks.

In our system, we prepare tenfold serial dilutions of the antigen to be detected (mouse antibody to apolipoprotein E, Biogenesis, Bournemouth, United Kingdom) in 0.1 M of bicarbonate coating buffer, pH 9.6. Then we immobilize the antigen on microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) in a volume of 50 μl at concentrations of 1 μg/ml to as little as 10 femtograms per milliliter (fg/ml) by incubation overnight at 4°C. Wells are then washed three times with 200 μl of phosphate-buffered saline (PBS), pH 7.4, and blocked with bovine serum albumin (BSA) (10 g/liter in PBS) for 2 hours. After three additional washings with PBS, biotinylated goat antibody to mouse immunoglobulin G (IgG) is added at 250 pg/ml in PBS that contains 0.01% (v/v) Tween 20 (PBS-Tween) and 1 g/liter BSA for 1 hour.

Avidin-biotinylated DNA complex is prepared by adding 30 μl of avidin preparation (component B of the ABC system from Vector Laboratories, Burlingame, California) and 5 μl of a biotinylated 479-bp PCR product, which encompasses apolipoprotein B complementary DNA (cDNA) nucleotides 10354 to 10832 (5), to 10 ml of PBS and then incubating the mixture for 30 min.

Unbound biotinylated goat antibody to mouse IgG is removed from the microtiter wells by five washings with PBS-Tween. Then 100 μl of avidin-biotinylated DNA complex is loaded and incubated for 30 min. Finally, wells are washed five times with 200 μl of PBS-Tween and three times with distilled water. A 50-μl PCR mixture

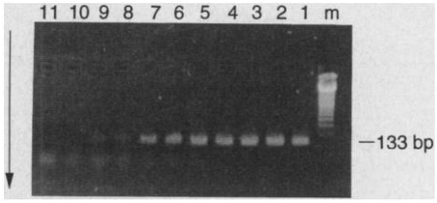

Fig. 1. Detection of antigens with immuno-PCR using avidin. Microwells were coated with progressively decreasing antigen concentrations. The antigen was overlayered with a sandwich of biotinylated antibody, avidin, and biotinylated DNA. Immobilized DNA was amplified with the use of PCR. Reaction products were visualized in an agarose gel. Lanes 1 to 9, products derived from wells coated with 0.05 μg to 0.5 fg of antigen (stepwise tenfold dilutions). Lane 10, 1 μg/ml BSA. Lane 11, no antigen present. m, molecular size marker.

[see (6)] is added to each well and cycled 30 times in a water thermal cycler on a float-boat (Bio-Med, Theres, Federal Republic of Germany) (95°C for 10 s, 60°C for 10 s, and 72°C for 20 s). 8 μl of the reaction products (133 bp) are run in a 2% (w/v) agarose gel that contains ethidium bromide (0.5 mg/l) and TBE, and the gel is photographed with a Polaroid camera.

We could detect as little as 10 fg of anti–apolipoprotein E per milliliter of coating solution (that is, 0.5 fg total) (Fig. 1, lane 9). False positive signals are difficult to see even after a photographic exposure time of 10 s.

Sano *et al.* (*3*) used a recombinant streptavidin–protein A chimeric protein that binds to the detecting antibody and to the biotinylated DNA to be amplified. The streptavidin–protein A chimera created by Sano *et al.* (*4*) allows for exploitation of any unlabeled detecting IgG. However, the protein A portion of the chimera would probably bind to free Fc fragments of capturing antibodies that would be coated to microtiter wells in a common sandwich ELISA setting. This would increase background and lower specificity. In addition, the proprietary chimeric protein is not readily available, whereas most antibodies can be purchased in a biotinylated form or be rapidly biotinylated in the laboratory. We conclude that replacing biotinylated horseradish peroxidase or biotinylated alkaline phosphatase in the Vector ABC system or in a comparable system with a biotinylated DNA label that is amplifiable would facilitate the use of this highly sensitive method and would contribute to its refinement and standardization.

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REFERENCES

1. L. E. M. Miles and C. M. Hales, *Nature* **219**, 186 (1968).
2. R. K. Saiki *et al.*, *ibid.* **239**, 487 (1988).
3. T. Sano, C. L. Smith, C. R. Cantor, *Science* **258**, 120 (1992).
4. T. Sano and C. R. Cantor, *Bio/Technology* **9**, 1378 (1991).
5. S. W. Law *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8142 (1986).
6. V. Ruzicka, W. März, A. Russ, W. Gross, *J. Lipid Res.* **33**, 1563 (1992).

25 November 1992; accepted 12 February 1993

Response: We appreciate the opportunity to discuss several issues about immuno-PCR raised by Ruzicka *et al.* A primary advantage of their system is the commercial availability of all of the reagents. The streptavidin–protein A chimera (*1*) we used is not yet commercially available, but the expression vector pTSAPA-2 is available from our laboratory upon request. This vector allows the production of the streptavidin–protein A chimera by the use of simple expression and purification procedures (*1*).

Ruzicka *et al.* add conjugates of avidin and biotinylated marker DNA to wells containing antigen-biotinylated antibody complexes. The potential drawback of this method is the lack of homogeneity of the avidin-DNA conjugates, which would reduce the accuracy and reproducibility of the system. Because avidin is tetrameric (*2*), mixing avidin with marker DNA containing one biotin per molecule at ratios below saturation generates five different species of conjugates, which include avidin without DNA, avidin saturated partially with DNA, and avidin saturated with DNA. Only the partially saturated avidin functions in the assay. Binding of avidin without marker DNA to antigen-biotinylated antibody complexes reduces the overall sensitivity of the system.

As Ruzicka *et al.* point out, our system (*3*) would generate high background signals in common sandwich assay formats, because the protein A moiety of the chimera binds to immobilized primary antibody. Similar problems would be encountered in samples containing endogenous immunoglobulins. We have been attempting to solve these problems by modifying our original immuno-PCR system. One method involves performing sandwich assays using $F(ab')_2$ or Fab fragments of primary antibody, neither of which binds to protein A. Endogenous immunoglobulins can also be removed by capturing antigen by immobilized antibody fragments. However, a more attractive approach is to preconjugate the antibody and the biotinylated marker DNA with the chimera. Preconjugation can be performed by mixing the three components at appropriate ratios, because the chimera binds both biotin and immunoglobin G stoichiometrically (*1*). Such conjugates should not bind to immunoglobulins present in samples, because the IgG-binding sites of the protein A moiety are saturated with antibody, and few exchange reactions should occur during the short periods of time used in these types of assays. Alternatively, chemical conjugates of antibody and streptavidin that contain biotinylated marker DNA could be used without the possibility of exchange reactions. Neither avidin nor streptavidin can make homogeneous antibody-marker DNA conjugates. Mixing biotinylated antibody and biotinylated DNA with (strept)avidin produces aggregates, because multiple biotins are generally incorporated into each antibody molecule. An additional advantage of the preconjugation format is the reduction in the number of steps in the protocol. Procedures with fewer numbers of steps have obvious advantages for the applications in clinical diagnostics.

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REFERENCES

1. T. Sano and C. R. Cantor, *Bio/Technology* **9**, 1378 (1991).
2. N. M. Green, *Methods Enzymol.* **184**, 51 (1990); *Adv. Prot. Chem.* **29**, 85 (1975).
3. T. Sano, C. L. Smith, C. R. Cantor, *Science* **258**, 120 (1992).

25 January 1993; accepted 12 February 1993