Catalysing a fruitful future

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QUANTITATIVE GENETIC

Localization of a gene with POSITIONAL CLONING

Study of a human Autoimmune Pathology using rats as an Experimental Model

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Practical Work Thesis: Immunology and Genetics

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Introduction:

The origin of the project is to study an autoimmune (meaning the immune system of an organism acts against its own components), multigenic (suggesting the combination of multiple genes) and multifactorial (involving genetic and environmental factors) human disease, which causes an increase of immunoglobulin IgG1 (a type of antibody) rate in the serum.

The main goal of the project was to identified and to localized the position of the genes related to the pathology. To do so, we used positional cloning. This technique consists of localizing the *QTL (Quantitative Trait Locus), refining it, and identifying genes of interest knowing only their chromosomal localization. Positional cloning is used in conjunction with linkage analysis, which establishes a linkage between genes and serves as a way of gene-hunting.

There are many advantages to use experimental models rather than humans such as silencing and decreasing environmental and allelic variability. In other terms, using an experimental model allow us to control the environment of the lab rat, and to use inbreed homozygotes lines also called “pure breed”. Furthermore, homologous genes exist between rats and humans that enable us to use such experimental model to our study.

Therefore, we used Brown Norway (BN) and Lewis (LEW) rats as our experimental model. We obtain congenic lines by repeated crossing of homozygous LEW and BN. The Brown Norway rats have a high concentration of Immunoglobulin IgG1 and are said to be “sensitive”, whereas Lewis rats have a low rate of IgG1 and are said to be “resistant”. A high rate of IgG1 means a more sensitive organism with higher chances to be impacted by the pathology.

In this research, 205 **F2 rats were genetically and phenotypically studied to locate the primary QTL *Aiid2* present on chromosome 10 between D10Wox26 and D10Rat27 (27,80 centiMorgan cM of length), using linkage analysis. This allowed the creation of a genetic map of 240 microsatellites (a nucleotide sequence that short tandem repeat and that is localized between specific sequence, enabling the use of a specific primer for a genetic amplification by PCR).
We also evaluate the rate of IgG1 in all our lab rat.

The main goal of our TP is to reduce the chromosomal region containing the QTL on chromosome 10 using positional cloning in congenic lines to enable us to localize the genes responsible for the variation of IgG1 rate.

** QTL is a DNA region (composed of multiples genes) associated with a quantitative characters (phenotype). QTLs are mapped by identifying which molecular markers correlate with an observed trait.*

*** F2 rats have a (BNxLEW)x(BNxLEW) phenotypes*

Materials and Methods:

We used Brown Norway, Lewis and congenic rats' DNA as well as their serums for the biological materials. Congenic lines are strains in which a chromosomal region of a homozygous strain has been replaced by the same chromosomal region of another homozygous strain. As part of our study, we used LEW.c10BN meaning LEW strains carried a portion of BN on chromosome 10. So we had six congenic lines LEW (B, C, D, E, F, I, If) that are distinguished by their diverse portions of BN in chromosome 10.

We used genetics amplification and Sandwich ELISA test to study rat's DNA and serums. Genetic amplification of DNA sequences by polymerase chain reactions (PCR) is used to make many copies of a specific DNA segment. The PCR is divided into three steps and performed in a thermal cycler. First, the denaturation of DNA at a high temperature (95°C for 20 seconds), which gives simple-stranded DNA from double-stranded DNA. Then the reaction temperature is lowered for 30 seconds at 60°C (specific temperature of the primers) allowing the hybridization of primers to each of the single-stranded DNA templates, which delimit the DNA portion to amplify. The primers hybridization temperatures in a PCR varies to force the primers to fix on the single-stranded DNA. Finally the elongation by a DNA polymerase (Taq) for 30 seconds at 72°C.

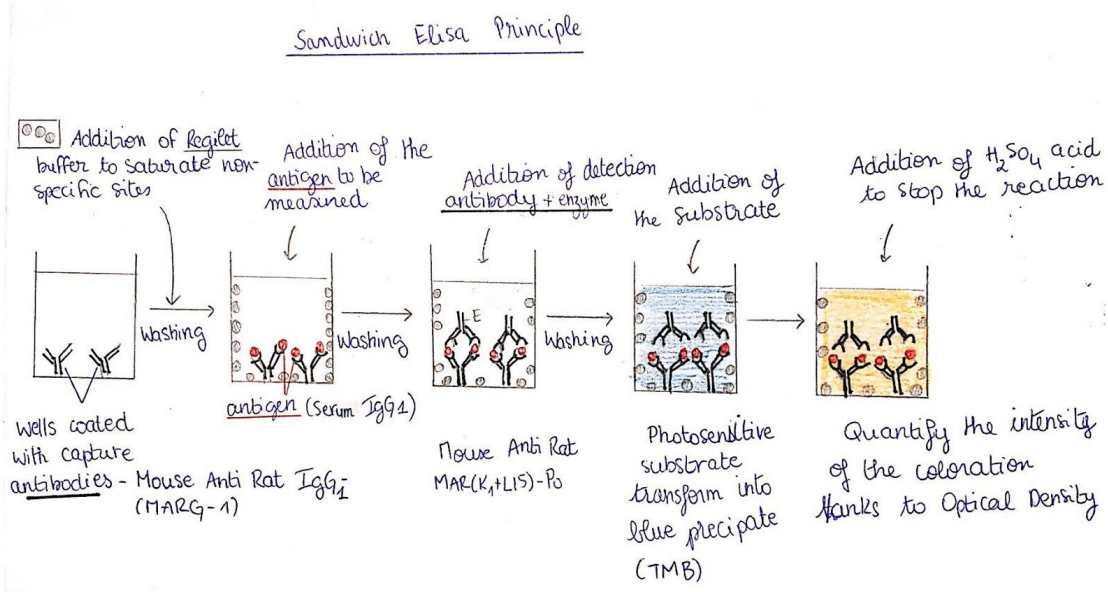
We made a mix of reagents from which we take the necessary volume for the eight samples to realize our PCR. Our PCR mix was composed of Taq polymerase which is a DNA polymerase enzyme able to resist the nucleic acid-denaturing conditions (95°C), required during PCR. In this experiment we used Taq Hot start which has been modified to start only at high temperature, hence a Taq activation step (at 95 ° C) in the PCR program before the repetition of 39 cycles. Taq polymerase comes from Thermus aquaticus bacterium that lives in hot springs where it is able to stand high-temperature level. Moreover, dNTP is one of the reagents of this mix which was introduced at 0,12 µL of nucleotides volumes. dNTP volume was determined using the coefficient from the final concentration to the volume per sample of MgCl2 reagent. Indeed, we already know the volume per sample and the final concentration of MgCl2.

MgCl2	:	2,5 mM = 2,5*10³ µM	→	1,5 µL
dNTP	:	200 µM chq	→	X µL

$X = (C_{final\ dNTP} * V_{MgCL2}) / C_{final\ MgCL2} = (200 * 1,5) / 2500 = 0,12\ \mu L$

In our mix we also added sucrose, which is used to increase the density of PCR products for electrophoresis DNA migration, as well as a color marker, Cresol Red, added to follow DNA migration during electrophoresis. Since those components don't interfere with the mix, they are useless for PCR. However we added them to gain time during our experiments.

We also performed a "Sandwich" ELISA test (enzyme-linked immunosorbent assay). An immunological ELISA test is used to detect and quantify an antigen sample in the serum, based on the specific recognition between antigen and antibody. In a Sandwich ELISA, the antigen of interest is sandwiched between two antibodies. We want to assay and quantify IgG1 in the serum of congenic lines.



Buffer Recipes

- Washing buffer: 40 mL of PBS (Phosphate-buffered saline) 10X QSP (quantité suffisante pour), 400mL H_2O, 4mL of Tween 20 $\frac{1}{10}$. Its role is to wash the wells between each step to remove non-fixed compounds.
- Overcoating buffer (milk protein): 10 mL of PBS 10X QSP, 100mL of H_2O, 5g of Régilait. It allows the saturation of nonspecific sites to specifically detect antigen.
- Dilution buffer: 20 mL of 10X QSP PBS, 200mL H_2O, 200µL Tween 20 $\frac{1}{10}$, 2mL of gelatin 10%. It allows the dilution of serums BN_1, BN_2, LEW_1, LEW_2, $Cong_1$, $Cong_2$. The range solutions A, B, C, D, E, F, G, H were prepared and deposited on the wells.

The concentration in the wells increases from lines A to H (Fig. 5). The average optic density of solutions A to H is used to make a standard curve. This curve will allow us to calculate the concentration of BN_1, BN_2, LEW_1, LEW_2, $Cong_1$, $Cong_2$ from their average optic density.

H has a 1/40 dilution factor and all of the other solutions have a 1/2,5 dilution factor.

To prepare 500 µL of H, we mixed 15µL of the initially given H_0 solution ($[H_0]$=1,51 mg/mL), and filled up with Buffer. We use 15µL because of the dilution factors : 1 → 40 ⇔ 15 → 500

To prepare all of the other solutions, we mixed 200µL of the solution previously made and filled up with Buffer. We use 200µL because of the dilution factors : 1 → 25 ⇔ 200 → 500

500 µL H → 15 µL H_0 + 585 µL Buffer	[H] = 377,5 ng/mL
500 µL G → 200 µL H + 300 µL Buffer	[G] = 151 ng/mL
500 µL F → 200 µL G + 300 µL Buffer	[F] = 60,4 ng/mL
500 µL E → 200 µL F + 300 µL Buffer	[E] = 24,2 ng/mL
500 µL D → 200 µL E + 300 µL Buffer	[D] = 9,7 ng/mL
500 µL C → 200 µL D + 300 µL Buffer	[C] = 3,9 ng/mL
500 µL B → 200 µL C + 300 µL Buffer	[B] = 1,5 ng/mL
A → blank	[A] = 0 ng/mL

The dilution factor of BN_1,LEW_1,$Cong_1$ is 1/200 and the dilution factor of BN_2,LEW_2,$Cong_2$ is ⅛.

To prepare 400 µL of BN_1 we mixed 2µL of the initially given BN_0 solution and filled up with Buffer. We use 2µL because of the dilution factors: 1 → 200 ⇔ 2 → 400

To prepare 500 µL of BN_2 we mixed 100µL of BN_1 and filled up with Buffer. We use 100µL because of the dilution factors: 1 → 5 ⇔ 100 → 500

400 µL BN_1 → 2 µL BN_0 + 398 µL Buffer

500 µL BN_2 → 100 µL BN_1 + 400 µL Buffer

We used the same volume to prepare LEW_1, LEW_2, $Cong_1$, $Cong_2$.

Results:

Every scientific result must be reproducible to be validated. That is why in our experiment we had 2 teams working on the same serum and microsatellites to ensure inter-team validity by small variability between results. Indeed, we had 2 teams working on the same primer for the electrophoresis and 2 teams working on the same congenic lineage serum for the sandwich ELISA test. So both teams were doing two identical but independent manipulations. Moreover, to ensure intra-team reproducibility of the experiment, we added duplicates of the same solutions in our ELISA test. (So A1 and A2 are filled with the same solution in Figure 5 ; Optic density analysis will allow us to calculate the variability between the identic solutions.)

Day 1, Genotypic results: PCR

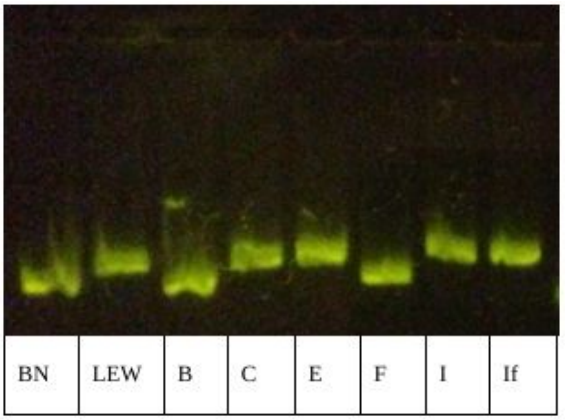


Figure 2 *: Agarose gel electrophoresis result for the primer Rat27 on the different congenic lineage and on Lewis and BN strains used as reference Team 13*

Primer	Team	BN	LEW	B	C	E	F	I	If
Rat42	1	NN	LL	NN	LL	LL	LL	LL	LL
Wox26	2	NN	LL	NN	LL	LL	LL	LL	LL
Mgh10	3 9	NN NN	LL LL	NN NN	LL LL	NN NN	LL LL	LL LL	LL LL
Rat172	4 10	NN NN	LL LL	NN NN	LL LL	NN NN	LL LL	NN NN	NN NN
Arb4	11	NN	LL	NN	NN	NN	LL	NN	NN
Wox13	Prof	NN	LL	NN	NN	NN	NN	NN	LL
Wox24	6 12	NN NN	LL LL	NN NN	NN NN	NN NN	NN NN	NN NN	LL LL
Rat27	7 13	NN NN	LL LL	NN NN	LL LL	LL LL	NN NN	LL LL	LL LL
Mgh4	8	NN	LL	LL	LL	LL	LL	LL	LL

Figure 3 *: Table of collective results of →*

Agarose gel electrophoresis in all the primers tested on all the different congenic lineage, and on Lewis and BN strains (N : BN ; L : LEW)

Team	BN	LEW	B	C	E	F	I	If
1 7	20,5 18,8	3,7 1,5	13,2 et 7,5 8,4					
2 8	21 18	1,1 1,6		1,2 1,7				
3 9	17 18	1,7 1,4			7 6,8			
4 10	9,4 15,5	1,3 1,3				1,2 1,5		
11 13	14,3 11,4	1,6 1,3					6,4 5,6	
6 12	13,6 16,3	1,4 0,9						5,5 4,5 et 9,2

Congenics lineages are BN between two microsatellites for B = [Rat42 ; Rat27] ; C = [Arb4 ; Wox24] ; E = [Mgh10 ; Wox24] ; F = [Wox13 ; Rat27] ; I = [Rat172 ; Wox24] ; If = [Rat172 ; Arb4] otherwise they are LEW

Day 2, Phenotypic results: ELISA test

←***Figure 4*** *: Table of collective results of Sandwich ELISA test representing the average initial concentration of IgG1 in mouse serum in mg/mL of all the different congenic lineage, and of Lewis and BN strains*

Team 13 studied with team 7 D10Rat27 primer and found exactly the same result. Team 13 and 11 found quite a similar level of IgG1 concentration in congenic mouse lineage of serum I. So both experiments are reproducible. The duplicate of the sandwich ELISA test made by team 13 had a low variability, so it is reproducible. To conclude, our manipulations are validated and successful because of the low variability between the reproductions of the same manipulations.

Conclusion and Discussion:

The electrophoresis shows that the DNA migrated at two different speeds between BN and LEW. We deduce that BN's and LEW's DNA have different alleles for the same gene. They also have a different density, that is why they migrate at different speeds. Because we can see a similar difference in the other samples, we are able to assimilate each sample to BN or LEW alleles for one microsatellite. With electrophoresis of all primers, we identified that congenic lineages are BN between two microsatellites : B = [Rat42 ; Rat27] ; C = [Arb4 ; Wox24] ; E = [Mgh10 ; Wox24] ; F = [Wox13 ; Rat27] ; I = [Rat172 ; Wox24] ; If = [Rat172 ; Arb4], otherwise they are LEW. We represented in black the BN region on Annex 1.

The sandwich ELISA gives us the concentration of IgG1 present in the serum of BN, LEW and Cong (B, C, E, F, I, If lineages) rats. The deeper yellow on the ELISA plate represents the solution with the higher IgG1 concentration. ELISA teach us if the congenic lineage is responder (BN phenotype's in the congenic) or non-responders (no change in the phenotype). A responder lineage tells us that the BN fragments contain the genes that control the IgG1 concentration. We identify B, E, I, If as responder lineages and C, F as non-responder lineages. We can use the conclusions of both experiments to reduce the QTL to 6,6 cM between D10Rat172 and D10Arb4 (Annex 5.b and Annex 6) corresponding to the smallest common region of all responders congenic lineages.

A questionable result in our study is the one of our PCR. Indeed, all concentrations of our PCR mix were overdosed due to improper use of the volumetric pipette. However, conclusive results were obtained in our PCR. We deduce that even with the improper uses of the pipette we must have put proportional volumes of products. So even if we didn't have the correct final volume we had the same concentration. Our experiments are validated by the reproducibility of our results which are comparable to Team 7 (same primer) and to Team 11 (same Cong).

Perspective:

Now that we succeed once at reducing the QTL using positional cloning, we could use the same method, again and again, to refine the QTL to a point where we can identify the genes responsible of the pathology.

In our experiment, we localized the region of interest and reduced it by precise mapping of the QTL to get to a small critical gap. We also need to make a physical map identifying overlapping clones covering the region to complete the research. This would enable us to make an inventory of overlapping clones of the chromosomal region. And finally, we could identify the mutation responsible of the disease (gene coding for the quantitative characters: IgG1 rate).

Now that we found one gene responsible of the multigenic disease, we could find the others using the same method. We could analyze the action of the protein coded by these genes to complete the research.

We also need to expand our result to Human to be able to find a cure to the pathology.

Picture bibliography :

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