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COVALENT COUPLING OF PROTEINS TO DIFFERENT STRAINS OF BACTERIA MODIFIED VIA SURFACE OXIDATION

OLGICA NEDIĆ*, R. LALIĆ*, B. LAKO** and JELICA BALAŽ***

*Institute for the Application of Nuclear Energy - INEP, Banatska 31b, 11080 Zemun, Yugoslavia, **Military Medical Academy - MMA, Crnotravska 17, Belgrade, Yugoslavia ***Institute for Plant Protection, Trg Dimitrija Obradovića 8, Novi Sad, Yugoslavia*

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Six different bacteria were investigated as protein carriers. Carbohydrate structures of the cell wall were oxidised and human IgG, as a model protein, was bound covalently through Schiff base linkage. The resulting bacteria - protein conjugates were characterised in terms of the amount of hIgG coupled per mass unit of bacteria. Agglutination reactions with sheep anti - hIgG antiserum illustrated the potential application of this protein immobilised on bacteria as a solid support.

Key words: bacteria, human IgG, solid support.

INTRODUCTION

Carbohydrate oxidation is a well known reaction involving transformation of vicinal hydroxyl groups, which can react with amino groups (Ferrier et al., 1972.). Any molecule having an amino functional group that is exposed, i. e. not sterically hidden, can bind to oxidised carbohydrate units. As proteins have several such amino groups, on average, they are readily immobilised on oxidised polysaccharide supports. Sephadex (Wilson et al., 1976.), Sepharose (Dean et al., 1985., Rapatz et al., 1988.) and microcrystalline cellulose (Dean et al., 1985., Al-Abdulla et al., 1989.) are most often used as oxidised protein carriers. The bacterial surface exhibits great structural analogy with polysaccharide supports. Carbohydrate units are major constituents of the cell wall, either as pure polysaccharide chains or in the form of lipo-and proteo- conjugates (Beveridge et al., 1991., 1992., Sleytr et al., 1992.).

Messner et al. (1992.) investigated different methods for the surface activation of bacteria. One of them was periodate oxidation. To oxidised bacteria trisaccharides were coupled and further used for immunisation. A detailed analysis of the oxidation of Brucella abortus was given in a previous paper. (Lalić et al., 1993.)

The present paper describes our experience in applying different oxidised bacteria as carriers for the covalent immobilisation of human IgG.

MATERIALS AND METHODS

Preparation of bacterial suspensions

The following bacteria were analysed: Brucella abortus, Bacillus subtilis, Erwinia amylovora, Erwinia carotovora, Pseudomonas syringae and Xanthomonas campestris. The last four are known as phytopathogenic bacteria. These bacteria differ in shape and size. As we had no experimental means to count precisely the number of bacteria, we used the nephelometric method of approximating bacterial numbers, (Kiraly et al., 1970.). The reference value was an O. D. (550 nm) of 0.23 for a suspension containing 8×10^8 bacteria/ ml. (Teodorescu et al., 1977.) The initial bacterial suspensions for these experiments were 38 - times more concentrated, i. e. about 3×10^{10} dead bacteria/ ml in 0.05 M PBS (pH 7.2).

Bacteria were killed chemically with phenol - saline solution (Lako, 1992.), prior to making the initial suspensions. In order to be more precise, we measured the constant dry weight (at 20^0C) of bacteria in each initial suspension and used these data in calculating the immobilisation effects. Interpretation of the results per mass unit of carrier is the usual way to describe the binding capacity of a solid support.

hIgG coupling to bacteria

The bacterial suspension (1.0 ml) was oxidised with 0.6 M $NaIO_4$ (1.0 ml) at room temperature (20^0C) for 6 h. After oxidation the suspension wascentrifuged at 6000 x g, for 10 min and the pellet was washed three times with PBS. Bacteria were resuspended in 1.0 ml PBS. Radioactive hIgG solution (1.0 ml) at the concentration of 10.0 mg/ml and containing 1×10^6 cpm/ml was added.

The reactants were incubated at room temperature for 18 h. Coated bacteria were separated from the solution at 6000 x g, during 10 min, and then washed three times with PBS. A working suspension (5.0 ml) was obtained in PBS - 0.01 % NaN_3 after discarding aggregates formed during the immobilisation, by centrifuging the suspension at 120 x g, for 5 min. Bacteria were stored at 40C until use.

hIgG (5μg) was labelled with ^{125}I (1 mCi, CIS, Gif sur Yvette, France) by the chloramine - T method. (Hudson and Hay, 1989.) ^{125}I - hIgG was used as a tracer in order to differentiate between the hIgG bound to bacteria in the working suspension from the hIgG bound to aggregated bacteria.

The amount of coupled hIgG was calculated as the difference between the initial and the unbound hIgG, measured spectrophotometrically. Radioactivity was detected on a gamma - scintillation - counter. The amount of bacteria was determined as constant dry weight at room temperature (20^0C).

Agglutination assay

A working suspension of bacteria (25 μl) was mixed with serially diluted sheep anti - hIgG antiserum (25 ml) in a microtiter plate. The antiserum titer was 3.26 g/l. The reactants were left overnight at 4^0C. The unspecific reactivity of the hIgG coated bacteria was tested with normal sheep serum.

hIgG coated bacteria in the reaction with anti - bacteria antisera

Rabbit antisera against Brucella abortus. *Erwinia amylovora* and *Xanthomonas campestris* were obtained in MMA laboratories. Unoxidised and hIgG coated bacteria in suspension (25 μl) were analysed in the agglutination reaction with specific anti - bacteria antiserum (25 μl).

RESULTS

Regardless of the strain of bacteria used, protein coupling to oxidised cells involved the formation of aggregates. In Table 1. the initial amount and the weight of bacteria in working suspension are shown.

Table 1. Dry weight of bacteria.

STRAIN OF BACTERIA	INITIAL SUSPENSION, WORKING SUSPENSION		
	(mg)	(mg)	(%)
B. abortus	6.1	3.0	49.2
B. subtilis	3.0	0.8	26.7
E. amylovora	6.8	3.1	45.6
E. carotovora	4.6	1.9	41.3
P. syringae	6.2	2.6	41.9
X. campestris	5.8	2.4	41.4

The oxidation reaction and protein immobilisation caused great loss of single bacteria from the suspension. In the working suspension 26. 7% to 49.2% of the initial amount of cells remained. The most significant decrease of bacteria was recorded from the *B. subtilis* suspension. B. abortus bacteria formed the smallest amount of aggregates.

The total amount of immobilised hIgG and its distribution between bacteria in a working suspension and aggregates are presented in Table 2.

Table 2. The amount of hIgG bound to bacteria.

STRAIN OF BACTERIA	TOTAL hIgG	hIgG DISTRIBUTTION (RATIO)*	hIgG IN WORK. SUSP.	
	(mg)		(mg)	(%)**
B. abortus	3.0	2.2 : 1	2.1	70.0
B. subtilis	2.8	1.1 : 1	1.4	50.0
E. amylovora	3.2	2.1 : 1	2.2	68.7
E. carotovora	2.0	2.0 : 1	1.3	65.0
P. syringae	3.0	1.7 : 1	1.9	63.3
X. campestris	3.3	1.8 : 1	2.1	63.6

* - hIgG distribution among bacteria in working suspension and in aggregates (ratio).
** - % of the totally bound hIgG.

The total amount of bound hIgG ranged between 2.0 mg and 3.3 mg (20.0-33.0 %) for the analysed bacteria. A more significant difference between bacteria was detected for hIgG distribution (20.0%). Thus 70.0 % of the hIgG that was bound to B. abortus (3.0 mg) remained in suspension, whereas the utilisation of hIgG immobilised on *B. subtilis* (2.8 mg) was only 50.0% in the working suspension.

Bacteria *E. carotovora* coupled the least hIgG on their surface (2.0 mg), while other phytopathogenic bacteria reacted similarly (3.0 - 3.3 mg of bound hIgG). In spite of the differences among phytopathogenic bacteria in the total amount of hIgG bound, the distribution of hIgG between the suspension and aggregates resulted in very close relative amounts of hIgG molecules that were not lost through aggregation (63.3 - 68.7%). By measuring the weight of bacteria in the working suspension and the amount of hIgG bound to them, it was possible to characterise each suspension with the amount of hIgG (in mg) bound per mass unit of bacteria (1.0 mg). We have termed this value the coating efficiency (Table 3).

Table 3. The coating efficiency of bacteria.

STRAIN OF BACTERIA	COATING EFFICIENCY (mg/mg)
B. abortus	0.7
B. subtilis	1.7
E. amylovora	0.7
E. carotovora	0.7
P. syringae	0.7
X. campestris	0.7

The values for the coating efficiency were 0.7 for *B. abortus, E. amylovora, E. carotovora, P. syringae* and *P. syringae* and *X. campestris* and 1.7 mg hIgG/mg bacteria for B. subtilis.

All the data obtained so far described quantitative parameters of the immobilisation process. The biological functioning of the coupled hIgG was investigated in an agglutination reaction with sheep anti - hIgG antiserum. The results of the agglutination test are given in Table 4.

hIgG molecules immobilised on *B. abortus, E. amylovora, E. carotovora* and *X. campestris* were recognised by specific anti - hIgG antibodies and exhibited no unspecific interactions with normal sheep serum. hIgG molecules immobilised on B. subtilis and *P. syringae* could not be detected in the described agglutination test. These bacteria remained dispersed in the plate wells, giving neither a positive agglutionation pattern nor a negative, sediment like, response. hIgG bound to *B. abortus,* displayed higher sensitivity in the agglutination test. Thus, anti - hIgG antibodies were detected in a greater antiserum dilution (1/1024), than when hIgG was bound to *E. amylovora* or *E. carotovora* (1/512), or to *X. campestris* (1/256).

Table 4. Agglutination test for hIgG detection.

hIgG CARRIER	THE GREATEST ANTI-hIgG ANTISERUM DILUTION AT WHICH VISIBLE AGGLUTINATION OCCURRED	NORMAL SHEEP SERUM
B. abortus	1/1024	—
B. subtilis	suspension remained dispersed	
E. amylovora	1/512	—
E. carotovora	1/512	—
P. syringae	suspension remained dispersed	
X. campestris	1/256	—

Rabbit antisera against *B. abortus, E. amylovora* and *X. campestris* were offered to complementary bacteria, untreated and hIgG coated, in the agglutination test. The results of these reactions are presented in Table 5.

Table 5. Agglutination test with specific anti - bacteria anti - sera.

STRAIN OF BACTERIA	THE GREATEST ANTI-BACTERIA ANTISERUM DILUTION AT WHICH VISIBLE AGGLUTINATION OCCURRED	NORMAL RABBIT SERUM
B. abortus	1/128	—
B. abortus - hIGg	1/64	—
E. amylovora	1/256	—
E. amylovora - hIgG	1/128	—
X. campestris	1/512	—
X. campestris - hIgG	1/256	—

All the bacteria investigated behaved in the same way. Untreated bacteria reacted with the specific anti - bacteria antiserum at greater limit dilutions than hIgG coated cells. The difference in each case was one dilution unit.

DISCUSSION

The general conclusion following from the results obtained is that bacteria, treated in the described manner, covalently bind large quantities of ligand - protein. The difference between various strains of bacteria is, however, significant. Protein coupling caused considerable loss of single bacteria in favour of aggregate formation. This side effect of the coupling process was noted by other workers, as well. Thus, Teodorescu et al. (1977.), Kleinman et al. (1978.) and Bratescu et al. (1980.) immobilised immunoglobulins to different bacteria which were treated with glutaraldehyde prior to protein binding. The final suspensions of protein coated bacteria were always centrifuged in order to separate aggregates formed during the coupling reaction.

In our previous paper (Lalić et al., 1993.) we described our attempts to reduce this side effect by changing the reaction medium (by using BSA or

TWEEN-20). However, the procedure could not be improved in this way. We also tried to sonificate the final suspension in order to disperse existing bacterial clumps. This treatment caused visual disappearance of aggregates, but after centrifugation at 120 x g, for 5 min, the same amount of bacteria was sedimented as in the procedure where the sonification step was omitted.

By using ^{125}I - hIgG as tracer, it was possible to determine the distribution pattern of hIgG between suspended and aggregated bacteria. The amount of aggregated bacteria, the total amount of bound hIgG and hIgG distribution seem to be specific values for a particular bacterial strain. These phenomena follow from the cell wall structure which is unique for each type of bacteria. The carbohydrate units of the cell wall contain a certain number of monosaccharides having vicinal hydroxyl groups. Not only the number, but the accessibility of these groups to reagent, as well as the distance between them, determine the number of ligand - molecules to be bound to the bacterial surface. Cells of *B. subtilis* coated with hIgG, for example, aggregated more easily (73.3% of the total amount) than other coated bacteria (58.7 - 50.8 %), withdrawing 50.0 % of the total hIgG bound. From the working suspensions of other bacteria 30.0 - 36.7% of the total hIgG was lost in the form of aggregates. The coating efficiency ranged from 0.7 to 1.7 mg hIgG/mg bacteria. This value, concerning the amount of hIgG bound per mass unit of bacteria, takes into account all the uniqueness of one bacterial type. It was interesting to note that all bacterial strains, except *B. subtilis,* exhibited the same coating efficiency: 0.7 mg hIgG/mg bacteria.

Messner and coworkers (1992.) have found that different strains of bacteria bind different amounts of ligand under the same coupling conditions. hIgG molecules bound to *B. abortus, E. amylovora, E. carotovora* and *X. campestris* were recognised by specific anti - hIgG antibodies. This immunological reaction confirmed that the immobilisation process did not cause structural deformation of hIgG, i. e. the antigenic determinants of hIgG remained preserved.

Although the coating efficiency of these bacteria was the same, the sensitivity of the agglutination test was different. hIgG molecules bound to *B. subtilis* and *P. syringae* could not be detected in the agglutination test. These two species of bacteria failed to agglutinate or to sediment. Therefore, the biological functioning of hIgG immobilised on *B.subtilis* and *P. syringae* should be determined in another immunological test. The agglutination test with specific anti - bacteria antisera was carried out in order to investigate if the immobilisation process changed the antigenic determinants on the bacterial surface. It was found that hIgG coated bacteria reacted with specific rabbit anti - bacteria antiserum up to one serum dilution less than untreated bacteria. This indicated that surface antigenic determinants on bacteria were only slightly deformed (modified) and/or masked during the oxidation and coupling process. The results obtained can be explained as the absence of free vicinal hydroxyl groups in the antigenic structure of bacteria against which the specific antibodies were raised. The other possible explanation is that even if free vicinal hydroxyl groups are present, they are sterically hidden. Also, most of the bacterial antigenic determinants were probably significantly separated from the bound hIgG molecules, so that the subsequent coating with hIgG was not an obstruction for antigen - antibody interaction.

It is known that some of the characteristic bacterial antigens are lipopolysaccharides (LPS). LPS of S - form of B. abortus (which was the form used in our work) is identified as the linear unbranched homopolymer of a 1,2-linked-4,6-dideoxy-4-formamido-D-mannopyrannosyl units. (Caroff et al., 1984., Zygmunt et al., 1991.) This structure contains no vicinal hydroxyl groups, so cannot undergo periodate oxidation.

The last information concerning the persistence of almost unaffected bacterial antigenic determinants after the coating process must be considered when choosing a bacterial species as a protein carrier. For that reason only non-pathogenic and phytopathogenic bacteria should be used, in order to avoid both infection of personnel and the crossreactions which may occur when dealing with animal and human sera.

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KOVALENTNO VEZIVANJE PROTEINA ZA RAZLIČITE VRSTE BAKTERIJA MODIFIKOVANE OKSIDACIJOM NJIHOVE POVRŠINE

OLGICA NEDIĆ, R. LALIĆ, B. LAKO i JELICA BALAŽ

SADRŽAJ

Ispitano je šest vrsta različitih bakterija kao nosača proteina. Ugljenohidratne strukture ćelijskog zida oksidovane su i humani IgG, kao model protein kovalentno je vezan uz stvaranje Schiffove baze. Dobijeni konjugati bakterija i proteina okarakterisani su količinom hIgG koja se vezala za jedinicu mase bakterija. Reakcije aglutinacije sa ovčijim anti - hIgG antiserumom ilustrovale su potencijalnu primenu proteina imobilizovanog na bakteriji kao čvrstom nosaču.